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# An Examination of Preimplantation Embryos and Endometrium Developed During Induced Aluteal Cycles in the Mare

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# AN EXAMINATION OF PREIMPLANTATION EMBRYOS AND ENDOMETRIUM DEVELOPED DURING INDUCED ALUTEAL CYCLES IN THE MARE

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This work is dedicated to my husband Carlos who was more than a supportive spouse during my studies, he was also a collaborator and colleague. He spent countless hours in the barn with my mares and me when he didn't have to. He was my shoulder to lean on when I needed it, and he always pushed me to do my very best work at all times. His encouragement and inspiration has helped me become the scientist I am today.

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## ABSTRACT

Changes to the embryonic environment that occur within the first few days of development can have significant effects on subsequent fetal development. The following studies investigate the effects of progesterone-deprivation during the early preimplantation period in the mare. The first study described embryo morphology (diameter, stage, and quality grade) collected from mares with induced aluteal cycles. The second study evaluated the effect of induced aluteal cycles on the future fertility of embryo collections as a potential method for cycle manipulation of embryo donor mares. The third study characterized embryonic and endometrial transcript expression of progesterone-mediated transcripts as well as those related to normal embryo growth and pregnancy establishment during induced aluteal cycles. Finally, the fourth study evaluated the potential for the successful establishment of pregnancy when preimplantation embryos were deprived of progesterone. Embryos developed in a progesterone-deprived environment were developmentally delayed by all parameters evaluated. A significant effect was seen on the transcript expression of embryos and endometrium collected from progesterone-deprived cycles in the mare. Embryos deprived of progesterone during the early preimplantation period demonstrated delayed embryonic vesicle growth compared to control cycles. No effect was seen on the future fertility of embryo collections after utilizing progesterone-deprived cycles. The results of these studies elucidate the significance of adequate progesterone levels beginning immediately post-ovulation in the mare.

# CHAPTER 1: GENERAL INTRODUCTION

## 1.1 General Introduction

The corpus luteum (CL) is a temporary endocrine structure formed on the ovary [1.1]. This structure plays an integral role in the establishment and maintenance of mammalian pregnancy. In the mare, this structure is the primary source of progesterone during the first one third of gestation and progesterone is detectable at high concentrations in maternal plasma (>10 ng/mL) [1.2, 1.3]. Progesterone plays a role in regulating uterine quiescence and nourishes the embryo prior to implantation by the production of histotroph [1.4, 1.5]. Adequate progesterone levels are required to maintain pregnancy and embryonic loss in the mare has been associated with plasma progesterone levels < 2 ng/mL [1.6]. The equine pregnancy is unique among mammals with an extended period of preimplantation embryo development prior to attachment and placentation [1.7]. Recent studies have investigated the effects of periovulatory doses of prostaglandin F2α (PGF2α) on luteal function, embryo development and pregnancy in mares [1.7-1.13].

## 1.2 Corpus Luteum

The preovulatory follicle undergoes many changes prior to ovulation in preparation for luteogenesis. During late estrus and prior to ovulation, the preovulatory follicle becomes less responsive to gonadotropins [1.14]. This results in a shift of steroid hormone secretion from estradiol to progesterone within the follicle [1.14]. After ovulation of the dominant follicle, the CL forms as a transient endocrine gland that maintains the luteal phase of the estrous cycle [1.1]. The rapid proliferation of capillaries that occurs during the early stage of CL development has been compared to the

vascularization of solid tumors [1.15]. At the time of ovulation, intense changes occur in the endothelium of vessels surrounding the follicle [1.15]. Within 48 hours a new complex network of vessels has developed and invaded the previously avascular granulosa cell layers, which induces luteinization and later nourishes the CL [1.15]. Several factors have been implicated in the regulation of CL angiogenesis including vascular endothelial growth factor, cytokines, tumor necrosis factor α, and interferon gamma [1.1, 1.16].

The CL forms on the ovary after ovulation. In most mammals the follicular theca and granulosa cells begin a rapid turnover to form the small and large luteal cells respectively [1.17]. The resulting large luteal cells are relatively unresponsive to LH and secrete high basal quantities of progesterone whereas small luteal cells secrete low basal quantities of progesterone and are responsive to LH [1.18, 1.19]. The histology of the equine CL differs as only the granulosa cells of the preovulatory follicle contribute to the fully formed equine CL [1.20]. In contrast to other species, the theca interna cells degenerate at ovulation in the mare and the cause for cell degeneration remains to be elucidated (apoptosis vs. necrosis) [1.20, 1.21].

In the absence of pregnancy, luteolysis occurs naturally at the end of diestrus to allow a return to estrus and chance for rebreeding. The luteolytic process requires multiple triggering factors working synergistically which include PGF2α, nitric oxide, and cytokines [1.22-1.25]. As a result, the CL undergoes both functional and structural regression; resulting in a reduction in progesterone production and changes in luteal tissue morphology. It has been demonstrated that apoptosis in the CL is essential in regulating cell number and is mediated by nitric oxide as well as cytokine receptors such

as Fas ligand [1.22]. A decrease in progesterone production has been described as a direct negative effect of PGF2α [1.26]. The endogenous luteolysin, PGF2α, is released from the endometrium in a pulsatile manner [1.27-1.29]. Furthermore, the secretion of PGF2α is stimulated by the pulsatile release of oxytocin, which is affected by the concentration of endometrial oxytocin receptors [1.30]. Endometrial oxytocin receptor concentration has been demonstrated to be lowest during estrus and highest on Day 14 in non-pregnant mares [1.30]. Additionally, oxytocin receptor affinity is lower in pregnant than non-pregnant mares [1.30].

The CL is a key component of diestrus and manipulation of the equine estrous cycle often centers on the presence or absence of a CL. Hormonal manipulation focuses on either the termination of or prolonging the lifespan of the CL. Exogenous progestagens have been administered to mimic the presence of a CL in an attempt to synchronize ovulation of a group of mares [1.31]. The success of this technique varies greatly according to the stage of the cycle and average follicle size when treatment is administered [1.32]. Conversely, termination of a CL is achieved by administration of exogenous PGF2α with reports of its use inducing luteolysis as early as the 1970s [1.33, 1.34].

**1.3 Prostaglandin F2 Alpha**

There are a several aspects of the reproductive physiology and endocrinology of the mare that makes them unique among large animals, one of which is the relationship between luteal blood flow and luteolysis [1.35]. Horses lack countercurrent exchange between the uterine vein and the ovarian artery compared to ruminants [1.36]. As a result of this unique anatomy, PGF2α must travel systemically from the uterus to the ovary in

mares vs. local action in ruminants where the uterine vein is closely associated with the ovarian artery [1.36]. Additionally, the plasma clearance of PGF2α is five times slower in mares compared to heifers, resulting in a longer half-life [1.37]. It has been described that the equine is approximately 18 times more sensitive to the luteolytic effects of systemic administration of PGF2α than the bovine CL [1.36, 1.38]. An in vitro study demonstrated that equine luteal cell membrane preparations had an affinity for PGF2α 10 times greater than bovine luteal cell preparations [1.39]. A combination of these factors; the high affinity of the mare CL to binding of PGF2α and the relatively slow metabolic clearance, account for the greater sensitivity of the mare CL compared to other domestic species [1.40].

The administration of PGF2α and its analogues have been utilized to induce luteolysis in mares for over 5 decades. For almost as long, it was believed that the CL was refractory to exogenous administration of PGF2α and luteolysis could not be induced prior to Day 5 [1.41]. Only recently have studies challenged this dogma. In the last two decades multiple studies have investigated the effects of periovulatory doses of PGF2α on luteal function and pregnancy rates in mares [1.7-1.12, 1.42, 1.43]. Several studies with varying single daily doses of PGF2α (cloprostenol) administered on Days 0, 1, and 2 post-ovulation had a transient effect on plasma progesterone concentrations [1.7, 1.11, 1.12]. In mares treated with 3 periovulatory doses of PGF2α, a decrease in plasma progesterone concentrations was observed, but levels rose to > 2.0 ng/mL by Days 4 and 5 in those studies [1.7, 1.11, 1.12]. Interestingly, a model has been developed which reliably prevents normal luteal function in all treated mares administered serial doses of PGF2α beginning within 12 hours of ovulation [1.8, 1.10]. This model demonstrates that

the early equine CL is responsive to PGF2α and normal luteal function can be completely interrupted if serial doses are administered during the early postovulatory period [1.8, 1.10].

**1.4 Progesterone**

Progesterone has long been considered the hormone essential for conception and establishment of pregnancy in mammalian reproduction. It has been demonstrated that in horses the CL is the exclusive source of progesterone during the first 150 days of gestation [1.3]. Sufficient progesterone concentrations are essential for maintaining pregnancy during the early postovulatory period between Days 2 to 5 [1.7]. High embryonic death occurs in the equine and it has been estimated that 30-40% of pregnancies are lost during the first 2 weeks following high fertilization rates [1.44]. The cause of early embryonic death is often unknown but has been attributed to hypoluteoidism [1.3, 1.45]. It has been described that increased embryo survival is more likely to occur when circulating progesterone concentrations are > 4 ng/mL compared to decreased survival when < 2 ng/mL [1.6].

Progesterone maintains uterine quiescence by reducing the number of gap junctions for uterotonic hormones, such as prostaglandin $F_{2\alpha}$ and oxytocin on the myometrium [1.46, 1.47]. The endometrium is primed by progesterone to produce the uterine histotroph, which is essential to conceptus survival and growth [1.48]. The histotroph contains a multitude of proteins that are essential for the survival, growth and development of the preimplantation embryo [1.49]. It has been demonstrated in sheep that the endometrial gland density is directly related to the survival and development of the conceptus [1.50]. Additionally, studies utilizing an ovine uterine gland knockout

model have demonstrated that functional endometrial glands and their secretions are necessary to ensure conceptus elongation and survival [1.51].

### 1.5 The Early Preimplantation Period

During the early preimplantation period there are again differences present in the equine compared to that of other domestic species. Implantation in the horse is protracted compared to other domestic animals; an extended period of conceptus development precedes implantation and placentation [1.51]. Due to the prolonged preimplantation period in the mare, adequate histotroph is especially important to nurture the embryo and maintain pregnancy [1.52]. The conceptus-initiated signal for maternal recognition of pregnancy (MRP) has yet to be elucidated in the mare compared to other domestic species. Certain factors have been found to be essential so the MRP signal can be released such the equine conceptus mobility throughout the uterus between Day 7 and 17 [1.53]. Additionally, an immunosuppressive protein called "early pregnancy factor," an extracellular form of heat shock protein 10, has been described in the mare [1.54, 1.55]. These factors ensure that MRP can properly occur to ensure continuation and secretory function of the CL beyond its normal lifespan [1.53]. This maintains the uterus in the appropriate progestational progesterone rich environment to support the developing pregnancy [1.53].

The equine embryo enters the uterus on Day 6, typically a morula at approximately 150 μm in diameter [1.56]. Between Day 6 to Day 8 the equine embryo rapidly grows and develops into a blastocyst then expands [1.57]. The embryo begins to form the capsule between the trophoblast and the inner surface of the zona pellucida [1.57]. Shortly after capsule formation is complete, the zona pellucida is shed from the

equine embryo [1.57]. The capsule is a structurally strong, acellular mucin-like glycoprotein matrix essential to pregnancy and protection of the embryo [1.58]. The lipocalin P19 produced by the equine endometrium is essential to the development of the capsule as it has been demonstrated that embryos developed in vitro do not develop a complete and cohesive capsule or shed their zona pellucida [1.59, 1.60]. Furthermore, it has been elucidated that P19 is mediated by progesterone and is one of the most abundant proteins found in the equine uterine histotroph [1.59].

**1.6 Scope of the Dissertation**

It has been described that progesterone production as early as Day 2 post-ovulation can have a significant effect on pregnancy outcome in the mare [1.7]. Furthermore, hypoluteoidism has been implicated as a factor of EED in the mare [1.3, 1.45]. The occurrence of EED each breeding season results in significant economic loss to the equine industry. The aim of the dissertation was to investigate the effects of progesterone-deprivation on the equine embryo and endometrium during the early preimplantation period in the mare. These studies may further elucidate the effects of hypoluteoidism on EED in the mare. The following series of studies utilized a novel in vivo model developed in our laboratory to prevent the formation of a functional CL immediately post-ovulation by administering serial doses of PGF2α beginning within 12 hours post-ovulation [1.8, 1.10]. Mares treated according to this protocol consistently have mean concentrations of plasma progesterone < 1.0 ng/mL (induced aluteal cycles) throughout the study period [1.8, 1.10].

The objective of the first study was to describe the morphology of embryos (diameter, stage, and quality grade) collected from mares with induced aluteal cycles.

The second study sought to characterize embryonic and endometrial expression of progesterone-mediated transcripts as well as those related to normal embryo growth, pregnancy establishment, and prostaglandin synthesis during induced aluteal cycles. The objective of the third study was to evaluate the difference in early pregnancy development when preimplantation embryos were deprived of progesterone. Finally, the fourth study sought to evaluate the effect of induced aluteal cycles on the future fertility of embryo collections as a potential method for cycle manipulation of embryo donor mares. The results of these studies are described in detail in the following chapters.

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# CHAPTER 2: MORPHOLOGICAL EVALUATION OF DAY 8 EMBRYOS DEVELOPED DURING INDUCED ALUTEAL CYCLES IN THE MARE[1]

## 2.1 Summary

A novel in vivo model utilizing serial administrations of PGF2α to induce aluteal cycles in the mare was used to evaluate the effects of progesterone-deprivation on the morphology of in vivo preimplantation embryos. We hypothesized that equine embryos produced during induced aluteal cycles (AL) would be developmentally affected, characterized by earlier embryo stage at collection, smaller embryo diameter, and lower quality grade, compared with those collected on the same day post-ovulation from control cycles during diestrus (high progesterone; > 4 ng/mL). Seven cyclic mares with a median age of 6.5 years (range 3-16) were utilized in a crossover design. Mares in estrus were artificially inseminated to a fertile stallion and randomly assigned to control or AL groups. Mares received either saline solution (control mares) or PGF2α (AL mares), twice daily on days 0, 1, and 2 and once daily on days 3 and 4. Serial blood samples were collected daily during estrus and until the day of embryo collection 8 days after ovulation. Mares were monitored until they returned to estrus, and artificially inseminated. Mares were switched to the opposite treatment group only after a successful embryo collection occurred during the previous cycle. Only cycles that produced embryos were used for analyses. No significant rise in progesterone was observed in the AL group with mean concentrations of plasma progesterone remaining < 1.0 ng/mL from ovulation until embryo collection on Day 8. This is in sharp contrast to the control

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(luteal) cycle where a post-ovulatory rise in plasma progesterone was observed. The mean daily concentrations of plasma progesterone were significantly higher in control vs. AL group beginning at Day 3 and remained so until Day 8. The mean (±SEM) embryo diameter of AL embryos was 171 ± 5 μm compared to 756 ± 99 μm for control embryos. The majority of the Day 8 AL embryos were classified as morulae (3/9) or early blastocysts (5/9) with only 2 embryos of quality grade 1 compared to the Day 8 control embryos that were mostly expanded blastocysts (6/7) with 5 of 6 being of quality grade 1. This study shows that serial administrations of PGF2α were able to prevent significant rises in plasma progesterone, thus inducing aluteal cycles characterized by a progesterone-deprived environment for developing embryos. Embryos collected from induced aluteal cycles were adversely affected as demonstrated by a lower quality grade, smaller diameter and earlier embryo stage at collection when compared to control embryos.

**2.2 Introduction**

There is strong evidence to support a crucial role for progesterone in regulating early pregnancy events in the horse, including preimplantation embryo development and embryo-maternal signaling for pregnancy. The correct maternal-embryo crosstalk during the preimplantation period is essential to establish pregnancy, specifically by ensuring corpus luteum maintenance and progesterone secretion [2.1]. Throughout the first 150 days of pregnancy in the mare, progesterone is detectable at high concentrations in the maternal plasma (> 10 ng/mL) and is produced by the primary and secondary corpora lutea (CL) of the ovary [2.2]. During the first 40 days of gestation in the mare, the primary CL is the sole source of progesterone [2.3]. One of the primary determinants of

an adequate environment conducive to the establishment of pregnancy is the continued supply of ovarian progesterone from the CL [2.1]. Progesterone is essential to provide the appropriate intrauterine environment for conceptus development [2.4]. Progesterone induces the production of endometrial histotroph, which is the primary conceptus nutrition until placentation [2.5]. The requirement for progesterone to drive uterine secretions necessary for conceptus development has been demonstrated in a variety of ways. For example, ovariectomized mares established and maintained pregnancy when administered only exogenous progesterone prior to the embryo transfer and during the first 100 days of gestation [2.6].

In addition to the production of endometrial histotroph, progesterone plays a role in the establishment and maintenance of early pregnancy by ensuring myometrial quiescence [2.7]. It reduces uterine contractility and the number of gap junctions and receptors for uterotonic hormones, such as prostaglandin $F_{2\alpha}$ and oxytocin on the myometrium [2.8, 2.9]. These are key functions for the initiation of maternal recognition of pregnancy (MRP) events in the mare. The events of MRP describe embryo-maternal communication during early pregnancy that prevents luteolysis to ensure ongoing progestational support that is vital for embryo development [2.10]. If proper MRP does not occur, early embryonic death (EED) may result. High embryonic death and significant economic loss occurs during early pregnancy in the equine. It is estimated that 30-40% of pregnancies are lost within 2 weeks following high fertilization rates [2.11]. The cause of EED remains largely unknown during the early period and has been attributed to a variety of factors. One of these factors may be hypoluteoidism, which is a condition characterized by low levels of progesterone incompatible to support early

pregnancy in the mare [2.3, 2.12]. It has been described that embryonic loss is more likely to occur when the circulating progesterone concentration is < 2 ng/mL compared to increased embryonic survival associated with progesterone concentrations > 4 ng/mL [2.13].

For the present study, we developed a novel model to induce aluteal cycles, characterized by a progesterone-deprived environment following ovulation. Administrations of serial doses of PGF2α beginning during the early post-ovulatory period have been shown to alter luteal development and function as evidenced by changes in concentrations of plasma progesterone [2.14-2.22]. Based on these effects, a series of studies in our laboratory refined a model to prevent the formation of a functional corpus luteum immediately post-ovulation (antiluteogenesis) [2.14]. In this model serial administration of PGF2α beginning within 12 hours post-ovulation (Day 0) was administered twice daily on Days 0, 1, and 2 and then once daily on Days 3 and 4 [2.14]. Mares treated according to this protocol consistently had mean concentrations of plasma progesterone < 1.0 ng/mL throughout (and after) the study period Days 0–5 post-ovulation [2.14]. In a subsequent proof of concept study, we have also reported that embryonic development could occur in mares that were artificially inseminated and underwent antiluteogenic treatment (serial administration of PGF2α) after ovulation [2.19]. Furthermore, embryos developing in this low progesterone environment could be "rescued" by the administration of exogenous progestin [2.19]. Therefore, this study was designed to evaluate the effects of progesterone-deprivation on in vivo embryos collected from progesterone-deprived cycles in the mare during the early preimplantation period, specifically days 0–8 post-ovulation. The objective of this study was to describe the

morphology of embryos (diameter, stage, and quality grade) collected from mares with induced aluteal cycles. We hypothesized that equine embryos produced during induced aluteal cycles (progesterone-deprived environment; < 1.0 ng/mL) would be developmentally affected, characterized by earlier embryo stage at collection, smaller embryo diameter, and lower quality grade, compared with those collected on the same day post-ovulation from control cycles during normal diestrus.

**2.3 Materials and Methods**

The Institutional Animal Care and Use Committee of Louisiana State University School of Veterinary Medicine approved all experimental protocols. The work was performed in a USDA-registered National Institute of Health-assured, and AAALAC International accredited animal facility in accordance with *The Guide for the Care and Use of Laboratory Animals* [2.23]. Seven cyclic Thoroughbred mares with a median age of 6.5 years (range 3-16) were utilized in a balanced crossover design from March to September 2016 in Baton Rouge, LA. Mares were randomly assigned to one of two treatment groups: control (normal diestrus) or induced aluteal (AL). Mares remained in the first assigned treatment group until a successful embryo collection. Immediately after the first successful embryo collection, they were assigned to the opposite treatment group and remained in that treatment group until a successful embryo collection. This study design resulted in the production of paired embryos from control and AL cycles from the same mare for analyses.

Mares were monitored by transrectal ultrasonography (SonoSite Edge, Fujifilm, Bothell, WA) throughout the study period. Mares in estrus as determined by the presence of uterine edema and with a follicle > 35 mm diameter were treated once with 2000 IU of

human chorionic gonadotropin (hCG) administered intravenously (Chorulon, Merck Animal Health, Kenilworth, NJ). Mares were artificially inseminated every other day until ovulation was detected with ≥ 1 x $10^9$ total motile spermatozoa from one stallion of known fertility. Reproductive examinations by ultrasonography were performed twice daily after insemination until detection of ovulation. After ovulation, mares were randomly assigned to one of two treatment groups: control (normal diestrus) or induced aluteal (AL). The AL mares were treated according to a protocol previously described [2.14]. Briefly, mares were treated twice daily with PGF2α (10 mg, IM, Lutalyse, dinoprost tromethamine, Zoetis, Florham Park, NJ) on days 0, 1, and 2, and once daily on days 3 and 4 [2.14]. The control group was treated on the same schedule with saline solution (2 mL, IM). Embryo collections were performed on Day 8 post-ovulation. Embryos were collected in an aseptic manner using Lactated Ringers Solution (LRS) with no supplementation (MWI Veterinary Supply, Boise, ID). The uterus was lavaged with 0.5 to 1 L of LRS at a time and an average 4 to 6 L of LRS was used in total. Recovered embryos were classified using a stereomicroscope (SMZ800, Nikon, Melville, NY) according to stage of development and assigned a quality grade on a scale of 1-4 [2.24]. Briefly, quality grade 1 was assigned to embryos with no abnormalities, spherical shape, uniform size, color and texture; grade 2 embryos had minor imperfections demonstrated by some extruded blastomeres, and slight irregularities in shape, size, color or texture; grade 3 embryos had moderate imperfections demonstrated by large percentage of extruded blastomeres, partial collapse of blastocele, or moderate shrinkage of trophoblast from zona pellucida; grade 4 embryos had complete degeneration and

embryonic death [24]. Additionally, embryos were photographed and their diameter measured (Nikon DSI-Fi2 camera with DS-L3 camera control, Nikon, Melville, NY).

Mares were monitored for a return to estrus by ultrasonography every other day after embryo collection. Once in estrus, mares were artificially inseminated and managed as described above. Mares remained in the same treatment group until an embryo was successfully collected. After a successful embryo collection, mares were assigned to the opposite treatment group. Only control or AL cycles with a successful embryo collection were used for statistical analyses.

Serial blood samples were collected from the time mares ovulated (Day 0) until embryo collection 8 days post-ovulation in both control and AL groups. Plasma was harvested and stored at -20° C until assayed for progesterone. Concentrations of plasma progesterone were determined by a progesterone radioimmunoassay (RIA). For the progesterone RIA, both the intraassay and interassay coefficients of variants were < 15%. A technician blinded to treatment protocols and groups performed the progesterone assays using a MP Biomedical double antibody approach as previously described [2.25].

Statistical significance for all data analyses was set at $P \leq 0.05$. Data for embryo diameter was analyzed using a one-tailed paired t-test and data for stage of embryo development and quality grade were analyzed using a one-tailed Wilcoxon signed-rank test. Concentrations of plasma progesterone were analyzed by ANOVA for repeated measures. All statistical analyses were carried out using Sigma Plot V12.5 (Systat Software, San Jose, CA). Data are expressed as mean ± SEM.

## 2.4 Results

A total of 7 successful embryo collections were recorded in both the control group and the AL group. A total of 7 embryos were collected from control embryo collections and a total of 9 embryos were collected from AL embryo collections as two mares had double ovulations in the AL cycles (Table 2.1). Overall the stage of embryo collected from control vs. AL cycles differed significantly ($p < 0.01$). In the control cycles, 86% (6/7) of embryos collected on Day 8 were classified as expanded blastocysts and 14% (1/7) were classified as blastocysts (Table 2.1, Fig. 2.1). In contrast, embryos collected from AL cycles on Day 8 were 33% (3/9) morulae, 56% (5/9) early blastocysts and 11% (1/9) blastocysts (Table 2.1, Fig. 2.1). Furthermore, the mean embryo diameter differed significantly in control vs. AL groups, 756 ± 99 μm vs. 171 ± 5 μm, respectively ($p < 0.001$) (Table 2.1, Fig. 2.1). Additionally, the quality grades of AL embryos (2 ± 0.26) was significantly lower than that of control embryos (1 ± 0.18), ($p < 0.05$) (Table 2.1).

No rise in plasma progesterone was observed in the AL group with mean concentrations remaining < 1.0 ng/mL from Day 0 until embryo collection on Day 8 (Fig. 2.2). This is in contrast to the control (luteal) group where a post-ovulatory rise in progesterone was documented (Fig. 2.2). The mean concentration of progesterone in the control group rose to 2.8 ng/mL on Day 2, in contrast to a mean concentration of progesterone of 0.7 ng/mL in the AL group (Fig. 2.2). The mean daily concentration of progesterone of mares in the AL group remained < 1.0 ng/mL throughout the duration of the study (Fig. 2). The mean daily concentrations of progesterone differed significantly between control vs. AL group beginning on Day 3 and remained so through Day 8 ($p < 0.001$) (Fig. 2).

Table 2.1 Developmental stage, quality grade, and diameter of embryos recovered Day 8 post-ovulation from control and induced aluteal cycles in each of the seven mares enrolled in the study.

| | CONTROL CYCLE | | | ALUTEAL CYCLE | | |
|---|---|---|---|---|---|---|
| **MARE ID** | **EMBRYO STAGE** | **QUALITY GRADE*** | **DIAMETER (µm)** | **EMBRYO STAGE** | **QUALITY GRADE*** | **DIAMETER (µm)** |
| **1** | Blastocyst | 1 | 360 | Early blastocyst | 2 | 180 |
| | | | | Early blastocyst | 2 | 175 |
| **2** | Expanded blastocyst | 1 | 1122 | Blastocyst | 1 | 195 |
| **3** | Expanded blastocyst | 1 | 663 | Early blastocyst | 2 | 188 |
| | | | | Early blastocyst | 2 | 175 |
| **4** | Expanded blastocyst | 2 | 600 | Morula | 3 | 151 |
| **5** | Expanded blastocyst | 1 | 884 | Morula | 1 | 151 |
| **6** | Expanded blastocyst | 2 | 660 | Early blastocyst | 3 | 161 |
| **7** | Expanded blastocyst | 1 | 1006 | Morula | 3 | 178 |

## 2.5 Discussion

The present study utilized a novel in vivo model to evaluate the effects of lack of progesterone on early embryo development in the mare. Progesterone-deprived (aluteal) cycles defined by mean plasma progesterone < 1.0 ng/mL were induced in all mares subjected to serial PGF2α treatments in the AL group. The serial administration of PGF2α beginning within 12 hours of ovulation had a significant effect on the developing corpus luteum prior to Day 3 post-ovulation. The absence of a significant rise in progesterone after ovulation in mares in the AL group indicated that early luteal cells are

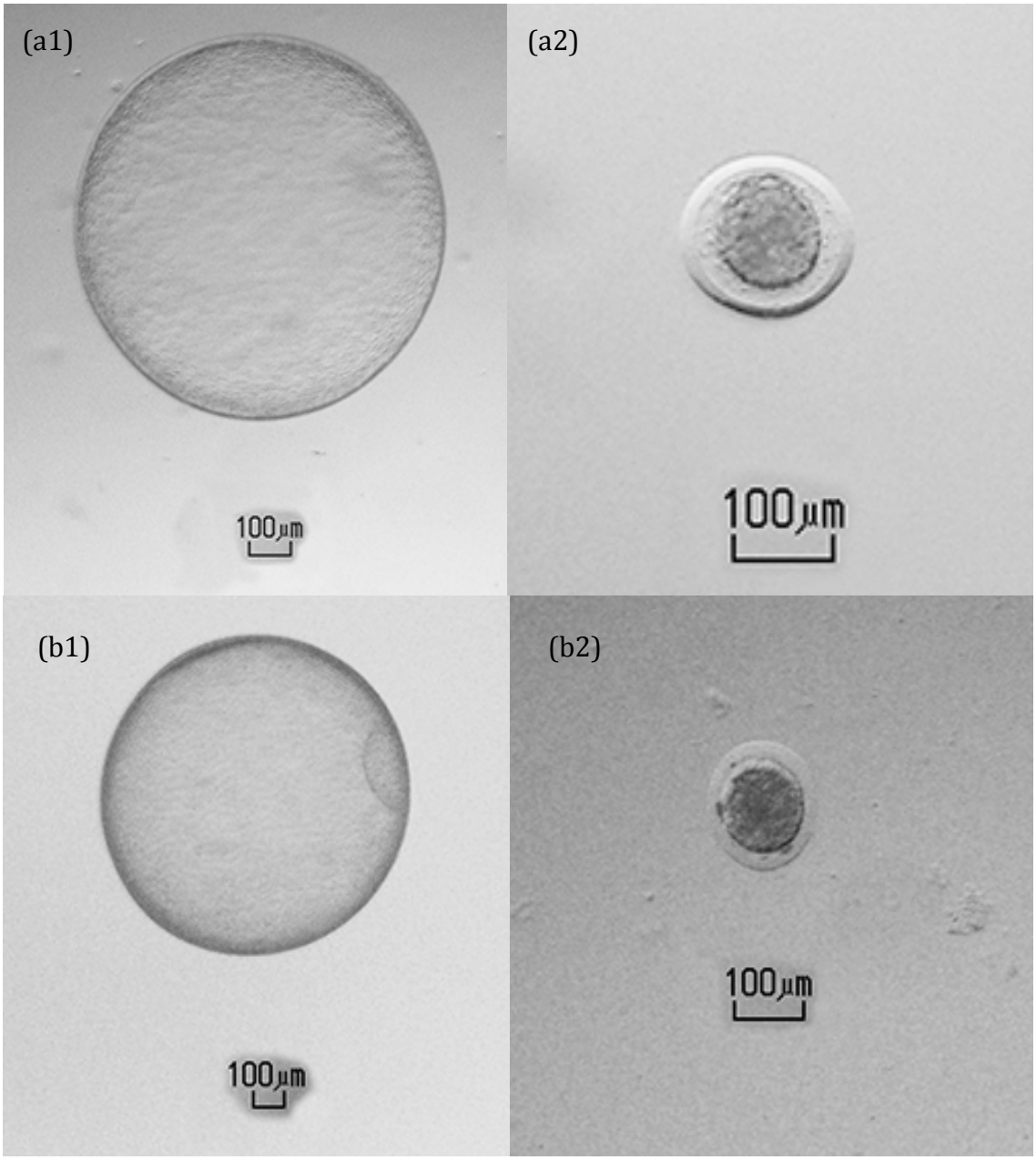

Fig. 2.1. Two pairs of embryos collected 8 days post-ovulation from control and induced aluteal cycles of two different mares. These embryo pairs illustrate the profound effect of progesterone-deprivation on embryo stage and diameter. Pairs of the same letter indicate the embryo was collected from control and induced aluteal cyles in the same mare. a1) An expanded blastocyst measuring 884 µm in diameter from a control cycle. a2) A morula measuring 151 µm in diameter from an induced aluteal cycle. b1) An expanded blastocyst measuring 1031 µm in diameter from a control cycle. b2) A morula measuring 178 µm in diameter from an induced aluteal cycle.

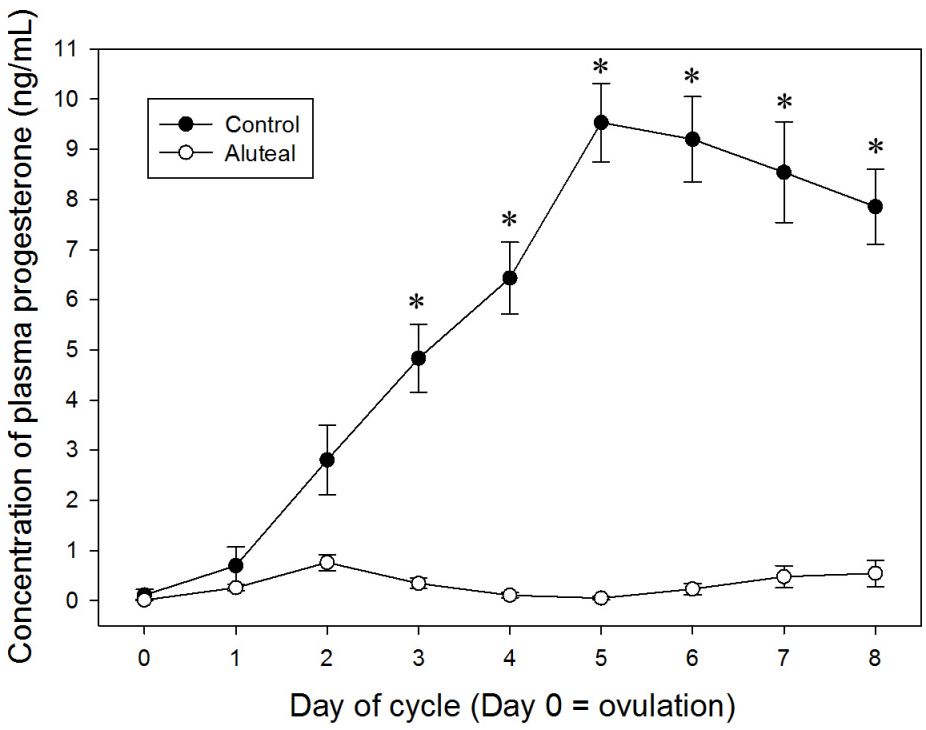


Fig. 2.2 Mean (± S.E.M.) daily concentrations of plasma progesterone in control and induced aluteal treatment groups. During the induced aluteal treatment, note that serial administration of PGF2α immediately post-ovulation twice daily on Days 0, 1, and 2, then once daily on Days 3 and 4 caused the mean daily concentrations of plasma progesterone to remain below 1.0 ng/mL. This is in contrast to the control (luteal) cycle where a post-ovulatory rise in progesterone was observed. The mean daily concentrations of plasma progesterone differed significantly in control vs. aluteal group beginning at Day 3 until Day 8 (*; $p < 0.001$)

being affected by $PGF_{2\alpha}$ much earlier than previously thought. The plasma concentrations of progesterone of aluteal cycles contrasted greatly to that of control cycles resulting in a clearly defined progesterone-deprived environment following ovulation in the mare. Additionally, the embryo quality and stage of development were adversely affected in Day 8 embryos collected from AL cycles when compared with Day 8 embryos collected from control cycles.

To our knowledge this is the first study describing in vivo embryo development in a progesterone-deprived environment from Day 0 to Day 8 followed by embryo collection and evaluation. The present study utilized a previously validated model administering serial $PGF_{2\alpha}$ treatments beginning within 12 hours of ovulation to reliably prevent normal luteal function (antiluteogenesis) in the mare [2.14]. Several studies have been conducted evaluating the effects of periovulatory doses of PGF2α on early luteal function and pregnancy rates in the mare [2.20-2.22]. These studies resulted in a transient interruption of early CL development when varying single daily doses of PGF2α (cloprostenol) were administered on Days 0, 1, and 2 post-ovulation as an approach to treat delayed uterine clearance in mares following breeding [2.20-2.22]. In those studies, concentrations of plasma progesterone did remain low for several days following ovulation in mares treated with three periovulatory doses of PGF2α administered once daily [2.20-2.22]. By days 4 and 5 after ovulation mares in those studies experienced resurgence in luteal function as concentrations of plasma progesterone rose above 2.0 ng/mL [2.20-2.22]. These results are in sharp contrast to the present study where serial administration of eight doses of PGF2α beginning within 12 hours of ovulation (Day 0) and continuing until Day 4 completely prevented the formation of a functional CL in all treated mares. As a result, aluteal cycles, as defined by mean plasma progesterone < 1.0 ng/mL, were induced in all mares throughout the study period when subjected to serial PGF2α treatments in the AL group.

In this study, the concentrations of plasma progesterone were significantly altered in the AL group where mean concentrations remained < 1.0 ng/mL from ovulation (Day 0) until embryo collection (Day 8). This contrasted drastically to the control group where

mean concentrations of plasma progesterone were > 2.0 ng/mL beginning on Day 2 and a typical diestrus rise was observed throughout the study period. Sufficient progesterone concentrations are crucial for maintaining pregnancy during the early stages of CL development [2.22]. The uterine histotroph is thought to be especially important for conceptus survival and development in sheep, cattle, pigs, and especially in horses, in which an extended period of preimplantation conceptus development precedes attachment and placentation [2.26]. Studies utilizing ovine uterine gland knockout model ewes have shown the importance of functional endometrial glands and their secretions to preimplantation conceptus development and survival [2.26]. Numerous proteins secreted by the equine endometrium and vital to the development of the conceptus are progesterone dependent. One of the most abundant proteins of the equine uterine histotroph is P19, a lipocalin important in the assembly of the embryonic capsule [2.27]. Due to the administration of multiple doses of $PGF_{2\alpha}$ beginning immediately post ovulation, we were able to completely prevent normal luteal formation (antiluteogenesis) as demonstrated by mean concentrations of plasma progesterone remaining < 1.0 ng/mL in all $PGF_{2\alpha}$-treated mares. The resulting progesterone-deprivation for the duration of the study likely had unfavorable effects on the development of uterine histotroph.

It has been demonstrated in several species that progesterone plays a key role in regulating gamete transport through the uterine tube. One aspect that makes the equine embryo unique is the prolonged transport through the oviduct lasting 6 days [2.28] Progesterone has been shown to play a crucial role in regulating ciliary beat frequency in vitro in the mouse, cow and human and in vivo in the guinea pig [2.29-2.32]. Regulation of ciliary motility is essential to facilitate the meeting of gametes and subsequent

transport of tubal stage embryos [2.29]. Additionally, the binding of progesterone receptor β in human oviductal epithelium affects smooth muscle relaxation, which is an important mechanism of oocyte transport from the ampulla to isthmus portion of the tube [2.30]. A progesterone-deprived environment may alter the day the embryo enters the uterus due to delayed transport through the uterine tube resulting in an altered embryo-maternal interaction.

When equine embryos enter the uterus they are typically a late morula measuring approximately 150 μm in diameter [2.33]. They rapidly develop from a morula to an expanded blastocyst one to two days after entering the uterus. A normal Day 8 equine embryo should be an expanded blastocyst, visible to the naked eye with a diameter of 500-1000 μm [2.33]. The Day 8 embryos collected in the control group displayed the expected characteristics of normal embryos. All of the control embryos collected except one were classified as expanded blastocysts and were between 600-1122 μm in diameter. This is in sharp contrast to the Day 8 AL embryos that were much earlier in development with approximately half of the embryos classified as early blastocyst and one third classified as morula with diameters between 151-195 μm. Furthermore, the quality of the AL embryos was significantly lower than the control embryos, indicating a negative effect of progesterone-deprivation on normal embryonic development.

In the equine embryo, coalescence of the endoderm colonies on the inside of the trophoblast at Day 8 completes yolk sac formation, which in turn initiates functional changes in the conceptus [2.34]. It has been described that the equine conceptus initiates steroidogenesis as early as Day 6 based on histochemical evidence of 3β-HSD [2.35]. However, stain intensities of 3β-HSD in Day 6 and 7 blastocysts are weaker with

measureable amounts of progesterone, androgens, and estrogens isolated from Day 8 blastocyst in the horse [2.35]. Once the yolk sac has completely formed, it secretes growth hormones, expresses proteins, and synthesizes and metabolizes steroids [2.34]. By Day 12 the equine conceptus produces significant quantities of estrogens and this activity continues to increase until Day 20 [2.35, 2.36]. It is likely that conceptus-derived estrogens, acting via endometrial estrogen receptors, do influence uterine function in a way that remains to be elucidated [2.37]. Interestingly, conceptus estrogens do not appear to regulate luteal maintenance in mares [2.38]. Additionally, it has been described that the Day 7 equine conceptus expresses mRNA for both intracellular progesterone receptors and membrane bound progesterone receptors [2.39]. It has been hypothesized that the reproductive steroids may exert their effects on the preimplantation equine conceptus directly, and not only via the endometrium [2.39]. The purpose of this study was to evaluate Day 8 embryos developed in a progesterone-deprived environment. Embryos collected in this study were collected on Day 8, the day of yolk sac completion and prior to the synthesis of significant levels of estrogens. Without a properly progesterone primed uterus secreting histotroph to provide adequate nutrients for the conceptus to develop and begin to secrete its own proteins and steroids, the events of MRP will likely be impaired [2.40]. Therefore, this model provides insight into the detrimental effects of hypoluteoidism on the endometrium and conceptus during the pre-implantation and MRP period in the horse. This novel in vivo model inducing progesterone deprivation during diestrus in the mare may offer the ability to further evaluate the direct effects of progesterone on the preimplantation equine conceptus and subsequent MRP events.

## 2.6 Conclusions

There is strong evidence to support a crucial role for progesterone in regulating early pregnancy events in the horse, including embryo development. The role of progesterone in the establishment of pregnancy in the mare is well described, but the specifics effects of progesterone deprivation on preimplantation embryo development have not been investigated. In this study, we highlight a novel in vivo approach to produce embryos in a progesterone-deprived environment (AL cycles) induced by serial administration of $PGF_{2\alpha}$ at ovulation. In all AL cycles the mean concentrations of plasma progesterone were < 1.0 ng/mL from ovulation (Day 0) until embryo collection (Day 8). The embryo quality was adversely affected and stage of development was retarded during the AL cycles. The production of aluteal embryos may serve as a novel in vivo model to investigate the direct and lasting effects of progesterone on preimplantation equine conceptuses. Further investigation applying this model can be utilized to investigate embryo-maternal interactions in the mare, especially those mediated directly or indirectly by luteal progesterone.

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# CHAPTER 3: EMBRYOS AND ENDOMETRIUM COLLECTED ON DAY 8 OF INDUCED ALUTEAL CYCLES IN THE MARE EXHIBIT ALTERED GENE EXPRESSION

## 3.1 Summary

Aluteal cycles were induced in the mare to evaluate the effects of progesterone deprivation on the gene expression of embryos and endometrium collected eight days after ovulation. We hypothesized that the transcript expression would be altered during induced aluteal cycles (AL) (low progesterone <1 ng/mL) when compared to control cycles during diestrus (high progesterone; > 4 ng/mL) for 1) the embryonic expression of progesterone mediated transcripts and those related to normal embryo growth and development and 2) the endometrial expression of progesterone mediated transcripts and those related to prostaglandin synthesis and normal pregnancy establishment. Seven cyclic mares with a median age of 6.5 years (range 3-16) were utilized in a crossover design. Mares in estrus were artificially inseminated to a fertile stallion and randomly assigned to control or AL groups. Mares received either saline solution (control mares) or $PGF_{2\alpha}$ (AL mares), twice daily on days 0, 1, and 2 and once daily on days 3 and 4. Serial blood samples were collected daily from Day 0 (ovulation) until the day of embryo collection and endometrial biopsy on Day 8. Mares were monitored until they returned to estrus and were artificially inseminated. Mares were switched to the opposite treatment group only after a successful embryo collection occurred during the previous cycle and only cycles that produced embryos were used for analyses. The study design resulted in paired samples from each mare for analyses. No significant rise in progesterone was observed in the AL group with mean concentrations of plasma progesterone remaining < 1.0 ng/mL from ovulation until embryo collection on Day 8. This is in sharp contrast to

the control (luteal) cycle where a post-ovulatory rise in plasma progesterone was observed. Real-time RT-PCR was utilized to evaluate the expression of *ESR1, PGR, CYP19A1, P19, SLC35A1, OCD, APOB, AQP3, NEU2* transcripts in the embryos and *PTGS2, P19, ESR1, HK2, sPLA2, PGR, CTGF, IFNE, FGF9, SLC36A2* expression in the endometrium. Four transcripts showed increased expressed in embryos developed during AL cycles *ESR1, P19*, *APOB* and *PGR* ($p<0.05$). Four transcripts showed increased expressed in endometrium developed during AL cycles *sPLA2, PGR, ESR1, FGF9* ($p<0.05$) and four transcripts showed decreased expression *P19, CTGF, IFNE, HK2* ($p<0.05$). Additionally, staining differences were present in endometrial staining for both ERα and PR receptor during AL cycles compared to control cycles. Embryos and endometrium developed in a progesterone-deprived environment during induced aluteal cycles demonstrated altered transcript expression. These results indicate that adequate progesterone levels may be a key mediator of the appropriate embryo-maternal environment during early preimplantation embryo development.
Keywords: equine, $PGF_{2\alpha}$, embryo, endometrium, progesterone, transcript

**3.2 Introduction**

A widely accepted principle of mammalian reproduction is that pregnancy cannot occur without progesterone [3.1]. In the mare, the first one third of gestation relies on progesterone secreted by the corpora lutea on the equine ovaries [3.1]. After ovulation, progesterone concentrations rise sharply in the mare and reach peak values of 12-20 ng/mL between Days 5 to 10 after ovulation [3.1]. The primary CL remains the only source of progesterone for pregnancy support during the first 40 days of gestation, but plasma concentrations begin to decline around Day 20 [3.1].

Early embryonic death (EED) occurs in the mare during the first 40 days of pregnancy [3.2]. This time period is divided into two phases, with the first period defined as day 0 (fertilization) to 13 and the second period from day 14 to 40 [3.2]. The incidence of EED during the first period in young fertile mares is reported to be 9% and can be as high as 73% in older mares or those with decreased fertility [3.2]. One of the factors involved in early embryonic loss may be hypoleutoidism, insufficiency of the CL, which results in low levels of progesterone during early pregnancy [3.1, 3.2]. Progesterone is a key modulator ensuring that the appropriate environment is maintained for establishment of pregnancy. It ensures uterine quiescence and the production of uterine histotroph, the primary conceptus nutrition until placentation [3.3-3.7]. It has been described that embryonic loss is more likely to occur when the circulating progesterone concentration is < 2 ng/ml compared to increased embryonic survival associated with progesterone concentrations > 4 ng/ml [3.8].

The preimplantation period is an essential period for the preparation of both the uterus and embryo for the establishment of pregnancy. Recently, transcriptomic studies of the equine endometrium have revealed a significant number of genes are differentially regulated in pregnant vs. nonpregnant mares between days 8 to 13.5 after ovulation [3.9]. Recently, several transcripts have been identified as developmentally important in the equine blastocyst [3.10]. Furthermore, several genes expressed in the preimplantation equine conceptus have been found to be upregulated during the course of normal pregnancy [3.11]. Some of these genes such as apolipoprotein B-100, clone CH241-268N6 and Fibrinogen beta chain precursor have been found to increase over 100 fold from Day 8 to Day 14 in equine conceptuses [3.11]. The preimplantation period marks a

critical window in which the developing embryo responds to the maternal environment by permanently modifying its epigenome [3.12]. The time period of early embryogenesis represents a window where the embryonic genome is exquisitely sensitive to disadvantageous developmental programming. This period of sensitivity appears to be concentrated into very early development in mammals, up to the blastocyst stage in mice and sheep [3.12].

It has recently been demonstrated that the equine preimplantation embryo expresses membrane bound steroid hormone receptors that exert their effects via non-genomic pathways [2.13]. Furthermore, it was elucidated that the equine embryo expresses progesterone receptors as early as Day 7 and the level of expression increases significantly between Days 7 and 10 [13]. Previous work in our laboratory has demonstrated that progesterone-deprivation during the early preimplantation period can dramatically affect the morphology of equine embryos collected on Day 8 [2.14]. However, the lasting effects of progesterone-deprivation on these embryos and their potential for pregnancy establishment have yet to be ascertained. One study evaluated the developing equine pregnancy during hypoluteal conditions by administration of PGF2α on either Days 12, 14, 16, or 18 post-ovulation [2.15]. Our laboratory has developed a novel in vivo model to investigate the effects of progesterone-deprivation during the early preimplantation period in the mare. In this model serial administration of PGF2α begins within 12 hours post-ovulation (Day 0), then is administered twice daily on Days 0, 1, and 2 and then once daily on Days 3 and 4 [2.14, 2.16]. Mares treated according to this protocol consistently had mean concentrations of plasma progesterone < 1.0 ng/mL throughout (and after) the study period of Days 0–5 and Days 0-8 post-ovulation [2.14,

2.16]. The application of this model consisting of 8 serial doses of PGF2α beginning immediately post-ovulation reliably prevents normal luteal function in the mare [2.14, 2.16].

The present study was developed utilizing our in vivo model to investigate the effects of progesterone-deprivation on the gene expression of preimplantation embryos and endometrium recovered from induced aluteal cycles in the mare. We hypothesized that the transcript expression would be altered during induced aluteal cycles (AL) (low progesterone <1 ng/mL) when compared to control cycles of normal diestrus (high progesterone; > 4 ng/mL) for 1) the embryonic expression of progesterone mediated transcripts as well as those related to normal embryo growth and development and 2) the endometrial expression of progesterone mediated transcripts as well as those related to prostaglandin synthesis and normal pregnancy establishment.

## 3.3 Materials and Methods

### *3.3.1 Animals and Sample Collection*

The Institutional Animal Care and Use Committee of Louisiana State University School of Veterinary Medicine approved all experimental protocols. The work was performed in a USDA-registered National Institute of Health-assured, and AAALAC International accredited animal facility in accordance with *The Guide for the Care and Use of Laboratory Animals* [2.17]. Seven cyclic Thoroughbred mares with a median age of 6.5 years (range 3-16) were utilized in a balanced crossover design from March to September 2016 in Baton Rouge, LA. Mares were randomly assigned to one of two treatment groups: control (normal diestrus) or induced aluteal (AL). Mares remained in the first treatment group assigned to until a positive embryo collection. Immediately after

the first positive embryo collection, they were assigned to the opposite treatment group and remained in that treatment group until a positive embryo collection. This study design resulted in the production of paired samples from control and AL cycles from the same mare for analyses.

Mares were monitored by transrectal ultrasonography (SonoSite Edge, Fujifilm, Bothell, WA) throughout the study period. Mares in estrus, as determined by the presence of uterine edema and a follicle > 35 mm diameter, were treated once with human chorionic gonadotropin (hCG, 2000 IU, IV, Chorulon, Merck Animal Health, Kenilworth, NJ). Mares were artificially inseminated every other day until ovulation was detected with $\geq 1 \times 10^9$ total motile spermatozoa from one stallion of known fertility. Reproductive examinations by ultrasonography were performed twice daily after insemination until detection of ovulation. After ovulation, mares were randomly assigned to one of two treatment groups: control (normal diestrus) or induced aluteal (AL). The AL mares were treated according to a protocol previously described [2.14]. Briefly, mares were treated twice daily with PGF2α (10 mg, IM, Lutalyse, dinoprost tromethamine, Zoetis, Florham Park, NJ) on days 0, 1, and 2, and once daily on days 3 and 4 [2.14]. The control group was treated on the same schedule with saline solution (2 mL, IM).

Embryo collections were performed on Day 8 post-ovulation. Embryos were collected in an aseptic manner using Lactated Ringers Solution (LRS) with no supplementation (MWI Veterinary Supply, Boise, ID). The uterus was lavaged with 0.5 to 1 L of LRS at a time and an average 4 to 6 L of LRS was used in total. Recovered embryos were identified using a stereomicroscope (SMZ800, Nikon, Melville, NY).

Embryos were washed ten times in lactated Ringers solution and immediately placed into 1.5 mL Eppendorf tubes (Fisher Scientific, Hampton, NH) in minimal media (2-10 μL) and snap frozen in liquid nitrogen. Samples were stored at -80° C until RNA isolation was conducted. Immediately after embryo collection, two endometrial biopsies were taken using alligator-jaw forceps. One biopsy sample was immediately placed in a 1.2 mL cryovial (Nalgene®, VWR, Radnor, PA) and snap frozen in liquid nitrogen. These samples were stored at -80° C until RNA isolation was conducted. The other biopsy sample was formalin fixed for a minimum of 24 hours. After being formalin fixed these samples were embedded in paraffin for histological analysis.

Mares were monitored for a return to estrus by ultrasonography every other day after embryo collection. When they returned to estrus, mares were artificially inseminated and managed as described above. Mares remained in the same treatment group until an embryo was successfully collected. Upon returning to estrus after a successful embryo collection mares were artificially inseminated and managed as described above and assigned to the opposite treatment group.

Serial blood samples were collected from Day 0 (ovulation) until 8 days post-ovulation in both control and AL groups. Ten mL of whole blood was collected in vacutainer tubes (BD Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ) containing lithium heparin with 20-ga 1-in vacutainer needles. Blood samples were immediately centrifuged at 3000 x g for 10 minutes. Plasma was harvested and stored at -20° C until assayed for progesterone.

### *3.3.2 Progesterone Assay*

Concentrations of plasma progesterone were determined by a progesterone radioimmunoassay (RIA). A technician blinded to treatment protocols and groups performed the progesterone assays using a MP Biomedical double antibody approach as previously described [2.18]. Concentrations of plasma progesterone on Days 0 to Day 8 were analyzed by ANOVA for repeated measures (Sigma Plot V14.0, Systat Software, San Jose, CA). Data are expressed as mean ± SEM and considered significant when $P \leq 0.05$.

### *3.3.3 Isolation of RNA*

Endometrial tissue homogenization was carried out individually using a Bullet Blender® Gold (Next Advance, Troy, NY) for 5 min at full speed. To perform homogenization tissues were placed in 1.5 mL Rino® tubes (RNase free, Next Advance, Troy, NY) with 8 stainless steel beads (RNase free, SSB14B, Next Advance, Troy, NY) and 500 μL of lysis buffer to achieve a 2:1:1 ratio of lysis buffer to tissue to beads. Total cellular RNA was isolated from individual homogenized endometrial samples using the Pure Link® RNA Mini Kit (12183018A; Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions. Isolated RNA was quantified via spectrophotometry using a NanoDrop One™ (Thermo Fisher Scientific, Waltham, MA) and analyzed for quality by determining RNA integrity number (RIN) using a Fragment Analyzer™ Automated CE System (Advanced Analytial Technologies, Inc., Ankeny, IA). Samples with a RIN of ≥ 6.8 were used for analysis and 12 samples had a RIN of > 8.

Total cellular RNA was isolated from individual Day 8 embryos using the PicoPure™ RNA Isolation Kit (KIT0202, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Isolated RNA was quantified via spectrophotometry using a NanoDrop One (Thermo Fisher Scientific, Waltham, MA) and analyzed for quality by determining RNA integrity number (RIN) using a Fragment Analyzer™ Automated CE System (Advanced Analytical Technologies, Inc., Ankeny, IA). Samples with a RIN of > 8 were used for analysis.

### *3.3.4 Real Time RT-PCR*

For individual embryo and endometrial samples, cDNA was synthesized using the SuperScript™ IV VILO™ Master Mix (Thermo Fisher Scientific, Thermo Fisher Scientific, Waltham, MA). Ten transcripts were chosen for evaluation in the Day 8 endometrium; prostaglandin-endoperoxide synthase 2 [*PTGS2*], P19 lipocalin [*P19*], progesterone receptor [*PGR*], estrogen receptor [*ESR1*], hexokinase 2 [*HK2*], phospholipase A2 [*sPLA2*], connective tissue growth factor [*CTGF*], interferon epsilon [*IFNE*], fibroblast growth factor 9 [*FGF9*], and solute carrier family 36 member A2 [*SLC36A2*]. Nine transcripts were chosen for evaluation in the Day 8 embryos; *ESR1, PGR*, aromatase [*CYP19A1*], *P19*, solute carrier family 35 member A1 [*SLC35A1*], ornithine decarboxylase 1 [*ODC1*], apolipoprotein B [*APOB*], aquaporin 3 [*AQP3*], and neuraminidase 2 [*NEU2*]. These transcripts were selected due to their involvement in steroidogenesis, embryo growth and development, and the establishment of normal pregnancy under luteal conditions. Primers were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), all products were less than 180 bp and are listed in Table 3.1. Primer specificity was confirmed through sequencing of the

Table 3.1 List of primers used in real-time RT PCR

| Gene name | Accession number | Sequence 5' --> 3' |
|---|---|---|
| *GAPDH* | NM_001163856.1 | F: TTGTCAAGCTCATTTCCTGGTATG |
| | | R: GTTAGGGGGTCAAGTTGGGAC |
| *FGF9* | XM_005601094.2 | F: GACGTAGCCCGTGCATCTTA |
| | | R: TCATCCCATCCGACCGTAGT |
| *CTGF* | XM_001503316.4 | F: TGCAGCAGCGTGAAGACATA |
| | | R: GTAATGGCAGGCACAGGTCT |
| *P19* | NM_001082509.2 | F: ATGAAGCACGGGAAGGAGAC |
| | | R: ACATCCACATGACATCCGGG |
| *PGR* | XM_001498494.4 | F: GTCAGTGGACAGATGCTGTA |
| | | R: CGCCTTGATGAGCTCTCTAA |
| *ESR1* | NM_001081772.1 | F: ACGATGCCACCAGACCATTT |
| | | R: CATGTGAACCAGCTCCCTGT |
| *sPLA2* | XM_005607005.1 | F: AGGCAGTCGCTTGGAAGTAT |
| | | R: CGGAAATCCAGCAAATGCCC |
| *HK2* | NM_001081776.1 | F: CTCAGAGCGGCTCAAGACAA |
| | | R: GCACACCTCCTTGACGATGA |
| *SLC36A2* | XM_005599277.2 | F: GCTTCTGCCACAGGCTTAAC |
| | | R: CCGGCTTTGAGTCCATACAT |
| *IFNE* | XM_005605033.2 | F: GCACTGGCCATTCTTCATGA |
| | | R: CTCCCAAGCATCCAGAGGAA |
| *PTGS2* | XM_005609749.1 | F: AAAAGATCCTGCCCCATCCG |
| | | R: CTCGCTGCAAGTCGTTTGAC |
| *NEU2* | XM_001499122.3 | TTTGGCAACTCTGGTGGGAC |
| | | GGATTCTGTAGGCATGGGCT |
| *SLC35A1* | XM_001500323.4 | CCGTCGTTAGTGTATGCCGT |
| | | CACCTGGTACACTGCTGCAT |
| *APOB* | XM_014730947.1 | GTCTACCAGTGCAACGACCA |
| | | TGCCTAAAGCGACCATTCGT |
| *AQP3* | XM_001917787.4 | TCGACCAGTTCATTGGCACA |
| | | GGGGTTGTTGTAGGGGTCAA |
| *CYP19A1* | NM_001081805.2 | TCCTCCCATCCCATTGTCCA |
| | | GTACCCAGATATCGGCCTGG |
| *ODC1* | XM_001502323.5 | CATGGGCGCTTATACTGTTG |
| | | GAAGTCGTGGTTCTGGATTTG |

resulting products. All PCR reactions were carried out in duplicate using Power™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Thermo Fisher Scientific, Waltham, MA) with a total volume of 10 µl containing 200nM of forward and reverse

primer and 1 µl of cDNA. A no-template control and no reverse transcriptase control (NRT) were included. Resulting Cq values were normalized for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) to obtain ΔCq values. The ΔΔCq method was used to calculate fold changes between treated and control samples. Normality of data was confirmed, and ΔCq values were analyzed using a paired t-test (Sigma Plot V14.0, Systat Software, San Jose, CA). Results were considered significant when $P \leq 0.05$.

### *3.3.5 Immunohistochemistry*

The formalin fixed specimens were processed, embedded and sectioned by routine histological methods. Sections from each specimen were examined by a board certified veterinary pathologist to define locations within the endometrium that contained representative areas of superficial surface epithelium and glands of the stratum compactum. These areas were marked on each slide. From the corresponding area on the original blocks, 2 tissue cylinders of 1-mm diameter were cored using a manual tissue microarrayer. The cores were arranged in the new microarray block at an approximate distance of 4 mm between each core. The top right corner of each microarray was left blank to mark the proper orientation.

Tissue Micro Array (TMA) sections were submitted for immunohistochemical staining for progesterone and estrogen receptor expression. TMA sections were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was performed by incubation in target retrieval solution (DAKO, Santa Clara, CA) at 125°C. The sections were incubated in 3% hydrogen peroxide in methanol, then blocked in a serum-free protein block (DAKO, Santa Clara, CA). The sections were then incubated

with anti-mouse primary antibodies for progesterone and estrogen receptors (PgR ab-8, prediluted, MS-298-R-7-A and ERα, 1:500 dilution, PA5-16440, ThermoFisher Scientific, Grand Island, NY). Optimal dilution was determined by titration on whole sections of equine control endometrial tissue. The sections were incubated with a biotinylated horse anti-mouse secondary antibody (1:200 dilution) for 30 minutes (Vector Laboratories, Burlingame, CA). Subsequently, the sections were incubated for 30 minutes with Vector RTU ABC Elite (Vector Laboratories, Burlingame, CA), followed by incubation with DAB+ chromogen (DAKO). Each incubation step was followed by rinse steps in Tris Buffered Saline (TBS) buffer. All sections were counterstained with hematoxylin.

Each TMA was scanned utilizing the Aperio slide scanning system (Leica Biosystems, Buffalo Grove, IL) at 40X magnification. Following the scanning procedure, region selection and staining intensity analysis was performed utilizing Aperio ImageScope software (Leica Biosystems, Buffalo Grove, IL). The free form pen tool was used to select three non-continuous areas of luminal endometrial epithelium (LE) and endometrial glands (GE) in each core. This resulted in 6 total areas of analysis for both LE and GE for each animal. Image analysis was performed using a Positive Pixel Count v9.1 algorithm with the following thresholds [Hue Value =.1; Hue Width =.5; Color Saturation Threshold =0.04; IWP(High) = 220; Iwp(Low)=Ip(High) = 175; Ip(low) =Isp(High)=100 Isp(Low) =0]. The percent positive pixels in a region was determined by adding the number of positive pixels (Np) to the number of strong positive pixels (Nsp) divided by the total number of negative and positive pixels (NTotal). The average staining intensity was determined by adding the total intensity of positive pixels (Ip) and

the total intensity of strong positive pixels (Isp) divided by the sum of the number of positive pixels (Np) and the number of strongly positive pixels (Nsp).

Normality of data was assessed, and percent positive pixels and average staining intensity were analyzed using a paired t test (Sigma Plot V14.0, Systat Software, San Jose, CA). Results were considered significant when $p \leq 0.05$ and data are expressed as mean ± SEM.

## 3.4 Results

### *3.4.1 Progesterone Assay*

The plasma progesterone profiles differed significantly between the control and AL groups. The mean daily concentration of plasma progesterone remained < 1.0 ng/mL throughout the study period (Day 0 to 8) in the AL group (Fig. 3.1). This is compared to the control group where a typical diestrus rise was observed and plasma progesterone peaked at > 9 ng/mL (Fig. 3.1). A significant difference was observed between the AL and control group beginning on Day 3 ($p < 0.001$) (Fig. 3.1). However, it is important to note that beginning on Day 2 concentrations of plasma progesterone had already risen to > 2.0 ng/mL in the control group compared to 0.7 ng/mL in the AL group (Fig. 3.1).

### *3.4.2 Real Time RT-PCR*

Evaluation utilizing real-time RT PCR demonstrated altered transcript expression when Day 8 embryos and endometrium are developed in a progesterone-deprived environment (< 1.0 ng/mL) during induced aluteal cycles. Eight out of the ten transcripts evaluated in the endometrium were differentially expressed in AL cycles compared to controls (Fig. 3.2). Four transcripts showed increased expression in endometrium

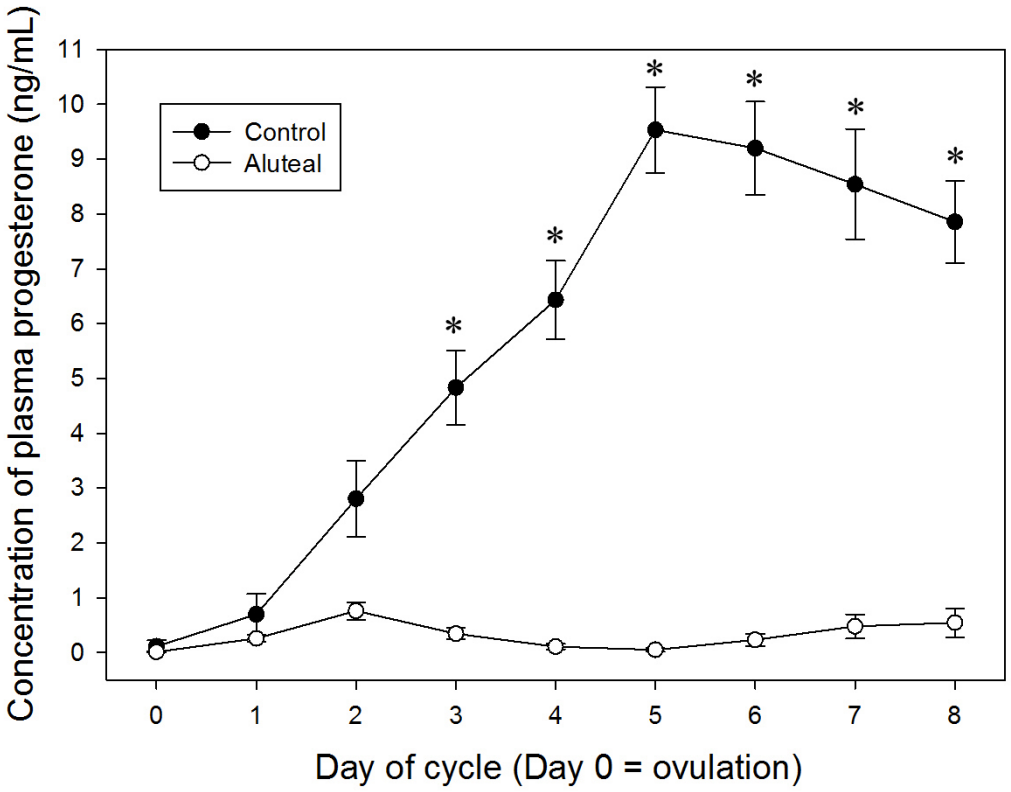


Fig. 3.1. Mean (± S.E.M.) daily concentrations of plasma progesterone in control and induced aluteal treatment groups. During the induced aluteal cycles the mean daily concentrations of plasma progesterone remained < 1.0 ng/mL. This is in contrast to the control (luteal) cycle where a post-ovulatory rise in progesterone was observed. The mean daily concentrations of plasma progesterone differed significantly in control vs. aluteal group beginning at Day 3 until the end of the study period (Day 8) (*; $p < 0.001$) developed during AL cycles compared to control cycles; *sPLA2, PGR, ESR1, FGF9* with

a 3.0, 2.5, 1.2, and 6.3-fold increase, respectively ($p < 0.05$) (Fig. 3.2). Additionally, *P19, CTGF, IFNE, HK2,* showed decreased endometrial expression when developed during AL cycles with a 0.03, 0.13, 0.12, and 0.14-fold decrease respectively ($p < 0.05$) (Fig. 3.2). Four genes were significantly upregulated out of the nine genes evaluated in Day 8 AL vs. control embryos (Fig. 3.3). In the Day 8 AL embryos *ESR1, P19*, *APOB,* and *PGR* exhibited a 1483.9, 93.2, 29.3, and 12.33-fold increase respectively ($p < 0.05$) (Fig. 3.3).

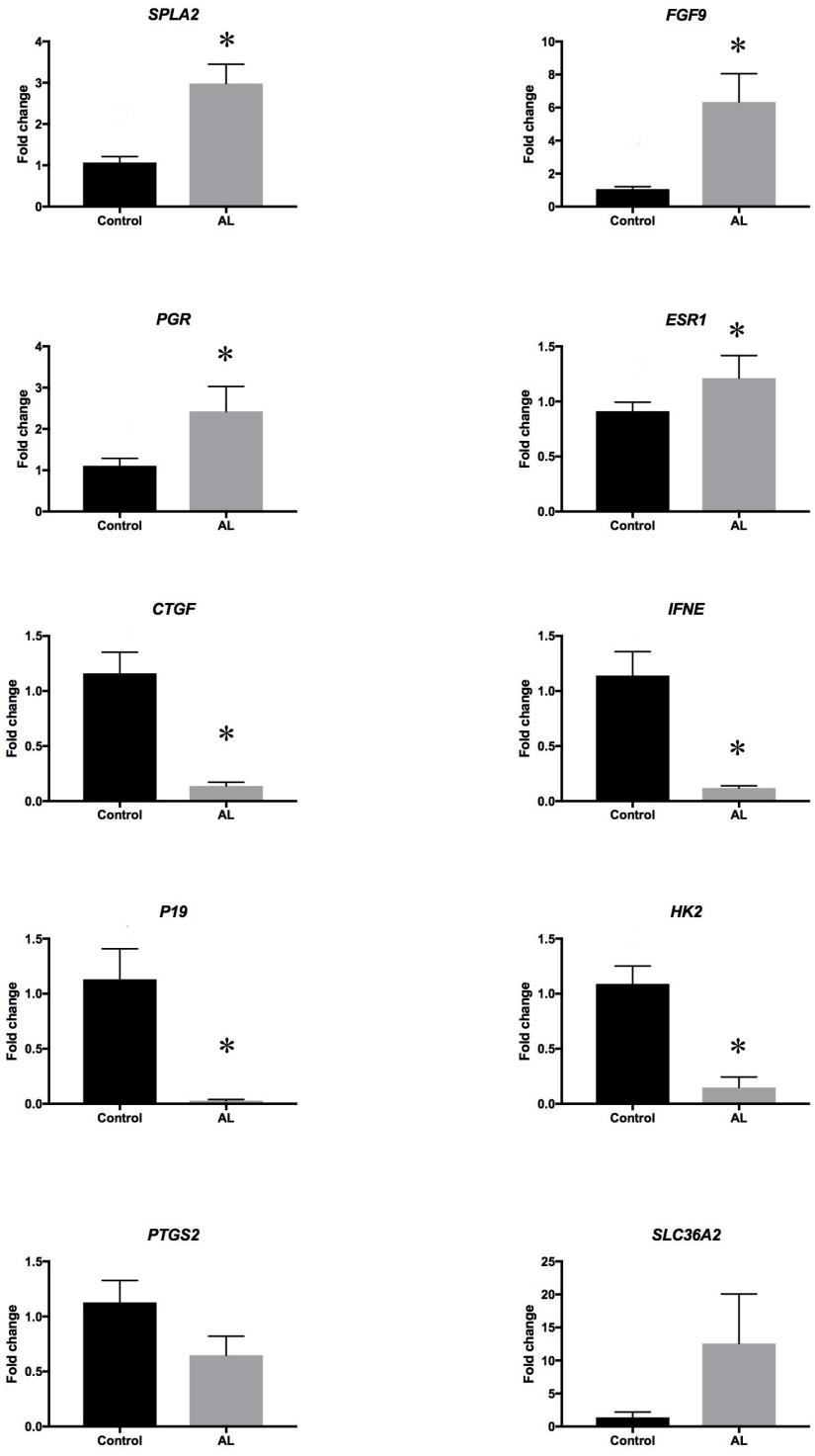


Fig. 3.2. Transcript expression in Day 8 control vs. induced aluteal endometrium. Eight out of ten transcripts evaluated were differentially expressed in the endometrium of induced aluteal mares vs. control. (*; $p < 0.05$)

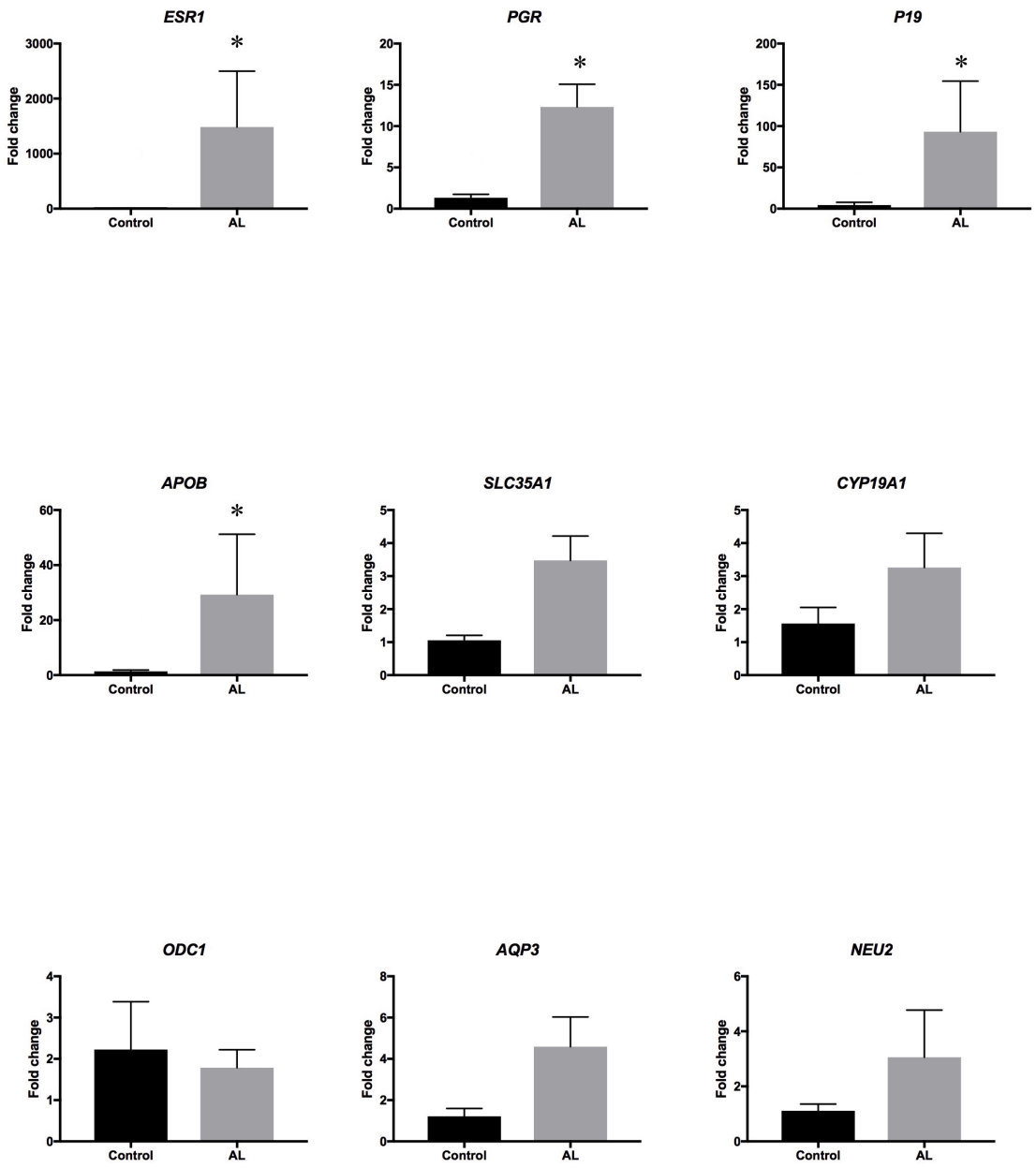


Fig 3.3. Transcript expression of Day 8 control vs. induced aluteal embryos. Four of the genes evaluated, *ESR1, PGR, P19* and *APOB,* were significantly upregulated in induced aluteal embryos vs. control (*; $p < 0.05$).

### *3.4.3 Immunohistochemistry*

The quantitative evaluation of Day 8 endometrium showed differences in both ERα and PR staining (Table 3.2). No difference was observed in ERα protein expression by percent positive pixels or average staining intensity in the LE. Conversely, in the GE,

a significant difference was observed in the average staining intensity of ERα protein ($p < 0.005$) (Table 3.2, Fig. 3.4). The average staining intensity of ERα in the control GE was 76.98 ± 2.15 compared to 59.94 ± 2.33 in the AL group ($p < 0.005$) (Table 3.2, Fig. 3.4). A significant difference was observed between the control and AL groups in both the percent positive pixels and average staining intensity of the PR in the LE ($p < 0.05$) (Table 3.2, Fig. 3.5). There was an increase in percent positive pixels in AL compared to control endometrium; 0.422 ± 0.21 vs. 0.234 ± 0.040, respectively ($p < 0.05$) (Table 3.2, Fig. 3.5). Additionally, the AL group demonstrated a decrease in average staining intensity, 63.77 ± 7.44 compared to 73.43 ± 8.68 in the control group ($p < 0.05$) (Table 3.2, Fig. 3.5). This is compared to the GE where a decrease in the percent positive pixels of the PR was observed in control vs. AL, 0.425 ± 0.017 vs. 0.634 ± 0.033, respectively ($p < 0.001$) (Table 3.2, Fig. 3.5). However, no difference was present in the average staining intensity of PR in the GE ($p > 0.05$) (Table 3.2, Fig 3.5).

Table 3.2 Immunohistochemical expression of progesterone receptor (PR) and estrogen receptor alpha (ERα) in the luminal (LE) and glandular epithelium (GE)

| | Percent postive pixels | | Average staining intensity | |
|---|---|---|---|---|
| | Control | AL | Control | AL |
| PR LE | 0.76 ± 0.03 | 0.75 ± 0.08 | 89.83 ± 3.72 | 79.77 ± 4.28 |
| PR GE | 0.88 ± 0.02 | 0.90 ± 0.02 | 76.98 ± 2.15[a] | 59.94 ± 2.33[b] |
| ERα LE | 0.23 ± 0.02[a] | 0.42 ± 0.02[b] | 73.43 ± 8.68[a] | 63.77 ± 7.44[b] |
| ERα GE | 0.43 ± 0.02[a] | 0.63 ± 0.03[b] | 57.94 ± 2.90 | 60.21 ± 1.94 |

a,b; $p < 0.05$

### 3.5 Discussion

The present study utilized induced aluteal cycles in the mare to evaluate the effects of progesterone-deprivation on the gene expression in Day 8 embryo and

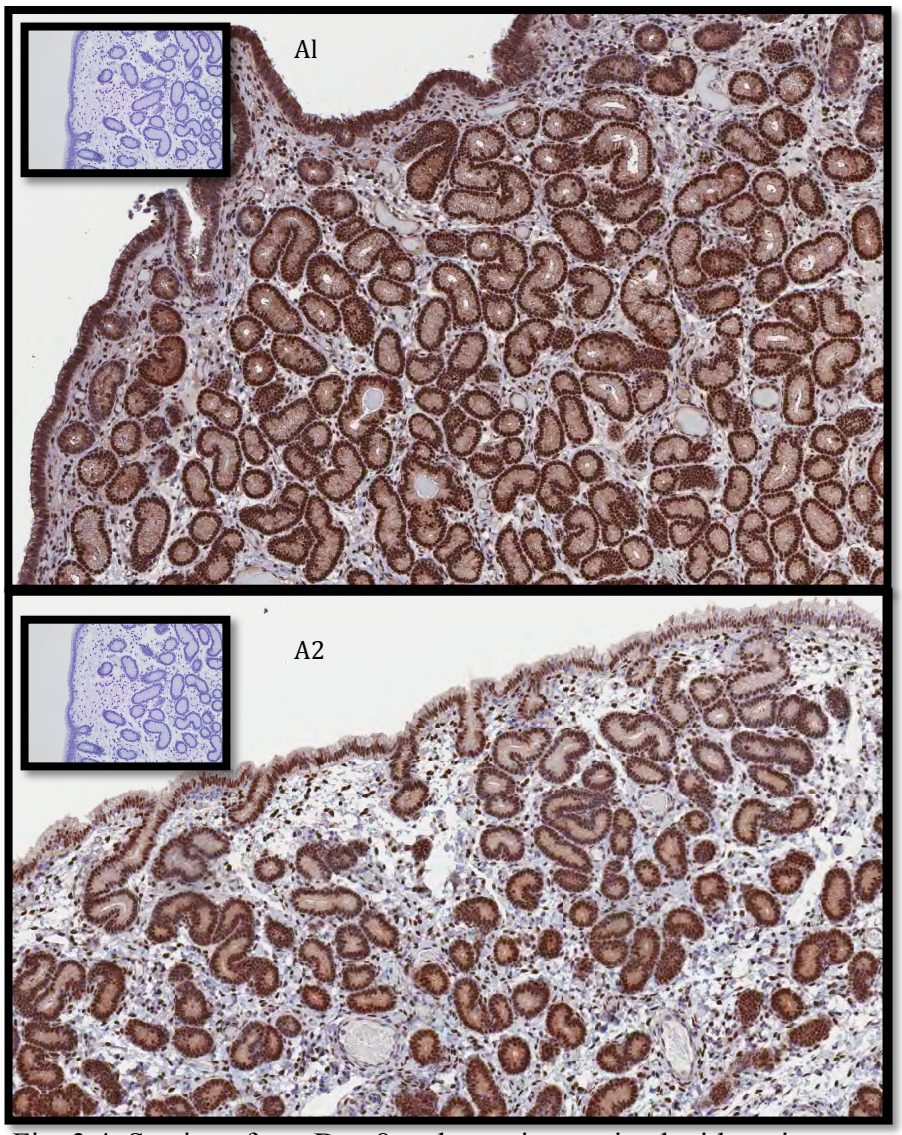

Fig. 3.4. Sections from Day 8 endometrium stained with anti-mouse primary antibodies for estrogen receptor alpha. Pairs of the same letter indicate the endometrium was collected from control and induced aluteal cyles in the same mare. A1) Control cycle; A2) Induced aluteal cycle. The negative control is inset in each panel.

endometrium. Progesterone-deprived cycles (< 1.0 ng/mL) were induced in all AL mares throughout the study duration by administration of serial doses of PGF2α beginning within 12 hours of ovulation. The concentration of progesterone differed significantly from that of control cycles, which displayed a typical diestrus rise. This altered hormonal milieu resulted in differential expression of transcripts selected for evaluation in both the

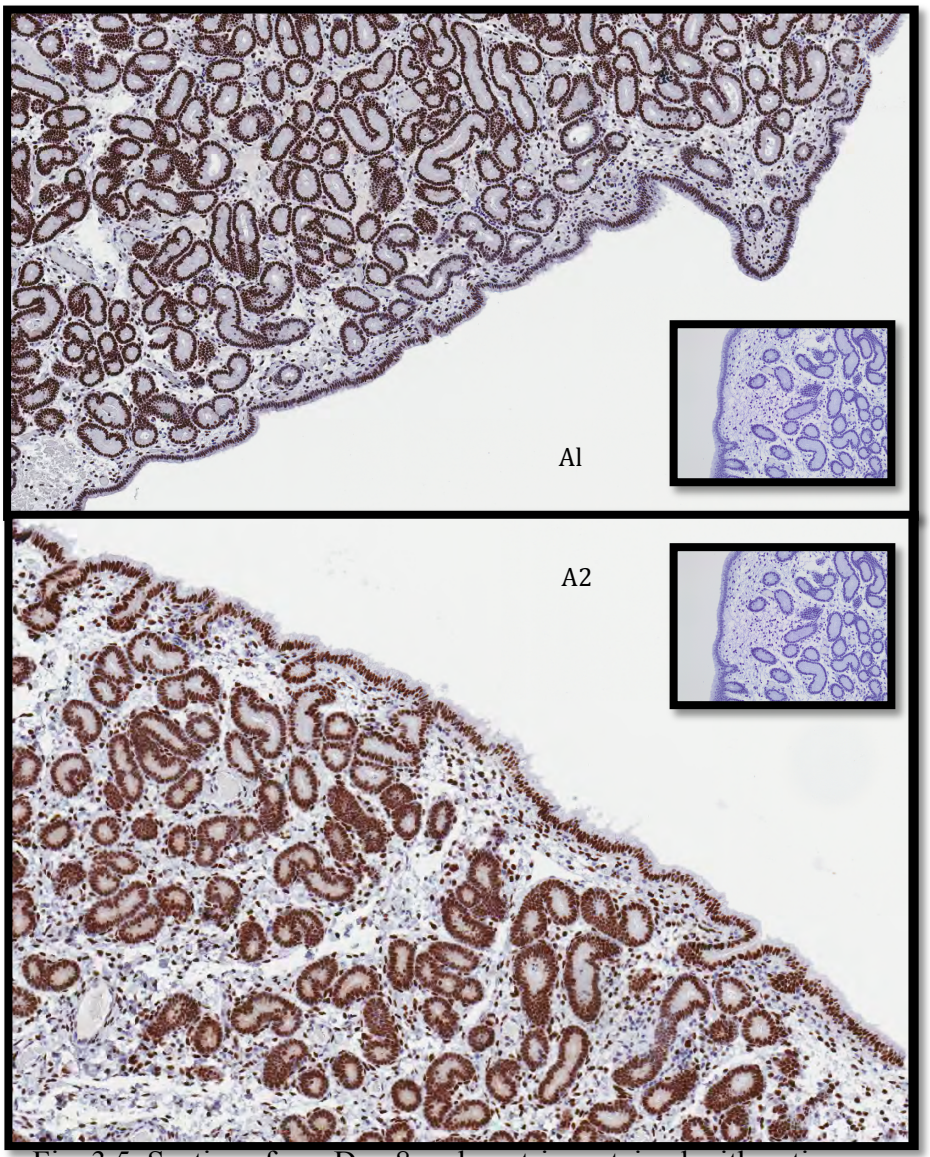


Fig. 3.5. Sections from Day 8 endometrium stained with anti-mouse primary antibodies for progesterone receptor. Pairs of the same letter indicate the endometrium was collected from control and induced aluteal cycles in the same mare. A1) Control cycle; A2) Induced aluteal cycle. The negative control is inset in each panel.

Day 8 embryo and endometrium. Additionally, the endometrial expression of PR and ERα protein differed in treatment mares.

Plasma progesterone remained < 1.0 ng/mL throughout the duration of the study in all treatment mares. A previously validated model utilizing eight serial injections of PGF2α beginning within 12 hours of ovulation was utilized to induce aluteal cycles in

treatment mares [3.14, 3.16]. This resulted in a progesterone-deprived in vivo environment during the early preimplantation period (Days 0 to 8), which was significantly different than control cycles with elevated plasma progesterone consistent with diestrus and early pregnancy. Progesterone is essential for appropriate embryo development during the early preimplantation period in mammals. It has been demonstrated in the cow and mare that progesterone concentrations are significantly higher in the oviduct ipsilateral to the ovary with a functioning CL during diestrus and early pregnancy [3.19, 3.20]. Furthermore, it has been demonstrated both in vitro and in vivo that the presence of progesterone can have rapid effects on ciliary beat frequency in the oviducts of humans, mice, cattle, and the guinea pig [3.21-3.24]. The ciliary beat frequency functions in transport of tubal stage embryos [3.22]. The effects of progesterone on the oviductal environment and embryo transport is especially important in the horse where embryo transport through the oviduct lasts 6 days [3.25]. Progesterone also plays an important role in the preparation of the uterus and endometrium for the establishment of pregnancy. Endometrial hypertrophy occurs during diestrus and increases with embryo implantation during pregnancy [3.26, 3.27]. Concurrent with endometrial hypertrophy, endometrial gland density increases and is directly related to successful pregnancy outcome in mares [3.28]. These endometrial glands synthesize and secrete uterine proteins and related substances, which comprise the histotroph, the primary conceptus nutrition until attachment and placentation [3.29]. The altered hormonal milieu present in AL cycles likely results in unfavorable effects on both the embryo and endometrium for the establishment of pregnancy.

Four transcripts were significantly higher expressed in embryos collected from AL cycles. These transcripts *ESR1, P19, APOB* and *PGR* are related to hormone signaling, embryonic capsule formation and nutrient transport. The increase in *ESR1* transcript, which codes for ERα, is likely due to the lack of progesterone during the AL cycles. It has been demonstrated that the expression of ERα is increased during estrus and decreased during pregnancy in the endometrium of mares [3.30]. The hormonal milieu of AL cycles mimics a mare in estrus with low progesterone versus the progesterone-dominated phase of early pregnancy. One of the most abundant proteins of the equine uterine histotroph is P19, a progesterone mediated lipocalin important in the assembly of the embryonic capsule [3.31]. One study has elucidated that the expression of P19 is highest in Day 8 equine conceptus and expression decreases from Day 10 to 14 [3.11]. In that study, the decreased embryonic *P19* transcript expression from Day 10 to 14 was contributed to the high amounts of P19 supplied to the conceptus of uterine origin [3.11]. The increased expression of *P19* in AL embryos may be due the low progesterone concentrations resulting in a lack of uterine P19 supplied to the conceptus. Apolipoproteins are proteins that play an essential role in the transport of lipids by binding lipids to form lipoproteins [3.11]. Embryos recovered from AL cycles are developmentally delayed compared to Day 8 embryos recovered from a typical diestrus mare [3.14]. It has been suggested that in the developing equine embryo, similar to the mouse, *APOB* may play a role in the transport of lipids via yolk sac mediated synthesis and secretion of lipoproteins [3.11, 3.32]. This increased expression of *APOB* may be attempting to compensate for nutrient deficiencies occurring in a progesterone-deprived environment. The Day 7 equine embryo expresses mRNA for both genomic and non-

genomic progesterone receptors [3.13]. As a result, it has been hypothesized that reproductive steroids, specifically estrogen and progesterone, may directly affect the preimplantation equine conceptus and not only act via the endometrium [3.13]. Our results demonstrating upregulation of *PGR* transcripts during induced AL cycles supports this hypothesis.

The expression of eight out of ten endometrial transcripts evaluated was altered in AL mares. Three of the four transcripts demonstrating an increased expression; *sPLA2, PGR, ESR1*, are progesterone mediated [3.30, 3.33, 3.34]. The PLA2 family is the rate-limiting enzyme in the biosynthesis of prostaglandins, and *sPLA2* is directly involved in the synthesis of $PGF_{2\alpha}$ [3.33, 3.35]. A negative correlation exists between PLA2 expression and progesterone levels, which indicates that progesterone controls the expression levels of the PLA2 family [3.33]. The increased expression of *sPLA2* during AL cycles relates to the negative association of progesterone levels and this transcript. The *ESR1* transcript is a steroid hormone receptor, which codes for ERα protein, and is involved in uterine development and steroid hormone activity [3.35]. In the mare, concentrations of *ESR1* are highest during estrus and early diestrus, which correspond to low levels of progesterone as seen in the AL mares [3.34]. The *PGR* transcript is progesterone-mediated and constant exposure to progesterone can cause a down regulation of transcript expression. Expression of *PGR* is higher during estrus than during pregnancy in the mare [3.30]. The expression pattern of both *ESR1* and *PGR* in the AL endometrium parallels the increased expression of these transcripts during estrus with low progesterone concentrations. It has been postulated that steroid hormones may drive regulation of the fourth transcript, *FGF9*. In the mare, the expression of *FGF9* is

upregulated on Day 12 in pregnant endometrium and this increased expression occurs shortly after the equine embryo begins to secrete significant amounts of estrogens [3.9, 3.36, 3.37]. The expression pattern of *FGF9* is complex, and upregulation of this gene corresponds to estrogen signaling in the pregnant endometrium of the pig, human and mare [3.9, 3.38-3.40]. This suggests that the expression of *FGF9* may also have a negative correlation with progesterone levels, which may explain the increased expression of *FGF9* in the progesterone-deprived AL cycles.

Four transcripts displayed a decreased expression in the endometrium of AL mares. The expression of two of these transcripts, *HK2* and *P19* has been demonstrated to be progesterone mediated [3.41, 3.32]. Glycogen, the main storage form of glucose in the body, is a component of the endometrial histotroph and *HK2* plays a role in glycogen synthesis by converting glucose into glucose-6-phosphate [3.41, 3.42]. The transcript abundance of *HK2* is significantly higher during diestrus and pregnancy than during estrus or anestrus in the mare [3.41]. Thus the expression of *HK2* is significantly greater during the progesterone-dominated phase of the estrous cycle [3.41]. As previously discussed, P19 is one of the most abundant constituents of equine histotroph and plays a role in the establishment of the embryonic capsule [3.31]. When evaluating the presence of immunoreactive P19, it is present in large amounts during diestrus and early pregnancy and barely detectable in the endometrium of mares in estrus [3.43]. *P19* is present in moderate amounts in the Day 8 conceptus and expression decreases from Day 10 to Day 14 due to the high amounts of uterine P19 supplied to the conceptus [3.11]. The expression of *P19* is progesterone mediated, and the decreased expression of *P19* in the progesterone-deprived cycles of AL mares agrees with previous reports describing a lack

of *P19* expression when progesterone concentrations are low such as during estrus. Type 1 interferons (IFNs) are a family of cytokines with antiviral activity [3.44]. Unlike other IFNs the expression of *IFNE* is not regulated by viral infection [3.44]. In humans and mice, the female reproductive tract constitutively expresses *IFNE*, and expression is hormonally driven in both species with highest expression during the estrogen dominated proliferative phase [3.44, 3.45]. Conversely, the equine endometrium expresses higher levels of *IFNE* during the progesterone dominated phases of diestrus and pregnancy compared to estrus [Klein C., unpublished data]. Furthermore, progesterone receptor binding sites have been identified in the promotors of both mouse and human *IFNE* genes [46]. Due to the unique hormonal regulation of this transcript, it is likely that the decreased expression of *IFNE* in the endometrium of AL mares is a result of the progesterone-deprivation during these cycles. Connective tissue growth factor is a transcript that has been associated with endometrial cell proliferation, differentiation and angiogenesis during early pregnancy in the mouse and mare [3.47, 3.48]. One study demonstrated that *CTGF* was expressed at high levels in Day 16 pregnant mare endometrium [3.47]. It is speculated that reciprocal signaling of *CTGF* and additional growth factors between the conceptus and endometrium assist in the maintenance of early pregnancy in the mare [3.47]. It has been described that embryos collected from AL cycles are developmentally delayed according to diameter, morphology and quality grade [3.14]. The resulting improperly primed endometrium and developmentally affected embryos produced during AL cycles likely affected the signaling events required for appropriate expression of *CTGF*.

In the present study, the percent positive pixels of PR in both the LE and GE were significantly increased in the AL mares. Our findings correspond with reports citing lack of localization of PR on the LE and GE in pregnant mares [3.49]. During early pregnancy PR are present at Day 10 and with continuous exposure to progesterone, these receptors are down regulated [3.49]. Additionally, it has been described that expression level of PR is greater during estrus than pregnancy in the mare [3.30]. The increase in percent positive pixels of PR in AL mares likely corresponds to the lack of progesterone resulting in an environment that mimics estrus. Additionally, this finding correlates with the increased expression of the *PGR* transcript in the endometrium of AL mares. The increased transcript expression likely resulted in an increase in translation and receptor expression in treated mares. Interestingly, the average staining intensity of PR in the LE was significantly lower in the AL endometrium. Average staining intensity reflects the concentration of PR in a given location. This reduced average staining intensity of PR in AL mares compared to the increased transcript expression of *PGR* may be due to the differential localization of PR within the nucleus and cytoplasm in the LE. In the present study the PR staining was subjectively localized predominantly to the nuclei of the epithelial cells rather than their cytoplasm. Several reports have described the localization of PR to the nuclei of the epithelial cells rather than their cytoplasm in the mare [3.30, 3.49]. The only difference noted in the expression of ERα was a decrease in average staining intensity in AL mares compared to control mares. However, it has previously been reported that immunolabeling of ERα is more intense during estrus than pregnancy [3.30]. The present results are the opposite of what would be expected with less intense ERα staining appearing in the progesterone-deprived endometrium. The decrease in the

average staining intensity of the ERα may be due to the localization of receptor in the GE. The localization of the ERα was similar to that of the PR with expression predominantly localized subjectively to the nuclei versus the cytoplasm. It has previously been reported that ERα is localized predominantly to the cytoplasm rather than the nuclei of the epithelial cells in the mare [3.49]. However, the variability in the staining pattern of ERα seen in this study compared to other studies may be due to differences in the primary antibody used. Interestingly, the decreased staining intensity of ERα in AL endometrium conflicts with the increased transcript expression of *ESR1* in AL endometrium. However, transcript expression only partially explains protein expression and different post-transcriptional regulatory factors regulate mRNA protein expression [3.30, 3.50]. The analysis of specific distributions of PR and ERα are not within the scope of this study.

**3.6 Conclusions**

In the mare adequate progesterone concentrations are essential for the successful establishment of pregnancy. Hypoluteoidism can result in inadequate progesterone concentrations incompatible with pregnancy and contribute to EED in the mare. Until recently, there was no model available to investigate equine pregnancy in hypoluteal conditions. This study utilized a novel in vivo model to induce aluteal cycles and evaluate the effects of in vivo progesterone-deprivation on embryos and endometrium. Several of the transcripts selected for evaluation were differentially expressed in Day 8 embryos and endometrium collected from AL mares. Additionally, the expression of selected steroid receptors was altered in the endometrium from AL cycles. The results of this study

indicate that adequate progesterone levels during the preimplantation period play an important role in regulating embryonic and endometrial gene expression in the mare.

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# CHAPTER 4: PROGESTERONE DEPRIVATION DURING THE PERIOVULATORY PERIOD CAUSES DELAYED PREGNANCY DEVELOPMENT IN THE MARE

## 4.1 Summary

Serial administrations of PGF2α were utilized to induce aluteal cycles in the mare to evaluate the effects of progesterone-deprivation on embryonic vesicle development in the mare. We hypothesized that progesterone-deprivation during early embryogenesis would result in delayed embryonic vesicle size and retarded growth compared to control cycles. Eight cyclic mares with a median age of 8 years (range 4 to 16) were utilized. Mares in estrus were artificially inseminated to a fertile stallion and randomly assigned to control or induced aluteal rescue (ALR) groups. Mares received either saline solution (control) or PGF2α (ALR), once daily on days 0 through 4. Then on Day 5 post ovulation mares in the ALR group were administered altrenogest. Serial blood samples were collected during estrus throughout the duration of the study period. Data are reported as mean ± S.E.M. The mean daily progesterone concentration from ovulation to altrenogest administration on Day 5 remained < 1.0 ng/mL in the AL group compared to the control group where concentrations rose > 2.4 ng/mL on Day 3 and were significantly different on Days 4 through 11 and Days 14 and 16. Pregnancy rate was significantly different with 50% in the ALR groups vs. 100% in the control group ($p < 0.05$). The embryonic vesicle size was significantly smaller on days 14 and 15 ($p < 0.05$) of pregnancy in the ALR compared to the control group. The delayed vesicle size in the ALR group did catch up to the control group and heartbeats were detected on either Day 22 or 23 in both groups. This study demonstrated that although embryo development and pregnancy can

occur in a progesterone-deprived environment normal growth and development may be altered.

**4.2 Introduction**

In mammals, progesterone is the solitary hormone required for the establishment and maintenance of pregnancy [4.1]. Progesterone is responsible for maintaining uterine quiescence and most importantly for the production of the uterine histotroph [4.2-4.4]. In the mare, the uterine histotroph contains many progesterone-mediated components such as P19, a lipocalin essential to the formation of the embryonic capsule [4.5, 4.6]. During the first 40 days of gestation in the mare a high rate of pregnancy failure can occur [4.1, 4.7]. This time period is divided into two phases, with the first period defined as day 0 (fertilization) to 13 and the second period from day 14 to 40 [4.7]. The incidence of embryonic loss during the first period in young fertile mares is reported to be 9% and can be as high as 73% in older mares or those with decreased fertility [4.7]. Early embryonic loss in the mare has a significant economic impact on the horse industry [4.7]. A variety of factors contribute to early embryonic loss, and these factors include hypoluteoidism, resulting in insufficient progesterone concentrations [4.1, 4.7]. Increased embryonic survival in the mare is associated with progesterone concentrations > 4 ng/ml [4.8].

Progesterone rises sharply after ovulation in the mare [4.9]. Due to this steep rise almost immediately after ovulation, progesterone is the predominant hormone present during early embryogenesis. Changes to the embryonic environment that occur within the first few days of development can have significant effects on subsequent fetal development [4.10]. The embryonic environment post-ovulation is of particular importance in the mare due to the extended period of embryo migration from the uterine

tube to the uterus, which lasts approximately 6 days [4.11]. Furthermore, studies have shown that the uterine tube ipsilateral to ovulation has elevated concentrations of progesterone in both the tissue and fluid [4.12]. Additionally, the mare also has a prolonged period of embryo migration and delayed implantation compared to other domestic species [4.13]. Appropriate uterine tube environment and proper priming of the uterus supported by adequate progesterone levels are essential for successful pregnancy establishment in the mare.

Studies in the mare have evaluated the importance of adequate progesterone production beginning immediately post-ovulation. Progesterone production from the newly formed CL beginning as early as 2 days post-ovulation can have a significant effect on the successful establishment of pregnancy [4.14-4.16]. One study has demonstrated that equine in vivo embryos produced in a progesterone-deprived environment are developmentally delayed [4.17]. The embryos produced were subjected to a continued period of progesterone-deprivation from Day 0 to 8 whereas other studies have involved only a transient loss of progesterone levels [4.14-4.17].

One question to answer is the viability of embryos subjected to an extended period of progesterone-deprivation beginning immediately post-ovulation, comparable to hypoluteoidism, and if these challenged embryos can establish a normal pregnancy. This study was developed to evaluate the viability of embryos developed during induced aluteal cycles characterized by plasma progesterone < 1.0 ng/mL in the mare. We hypothesized that embryos deprived of progesterone during the periovulatory period (Days 0 to 4) and then "rescued" by administration of altrenogest on Day 5 would be

developmentally delayed, as indicated by smaller embryonic vesicle diameter and delayed detection of a heartbeat.

**4.3 Materials and Methods**

The Institutional Animal Care and Use Committee of Louisiana State University School of Veterinary Medicine approved all experimental protocols. The work was performed in a USDA-registered National Institute of Health-assured, and AAALAC International accredited animal facility in accordance with *The Guide for the Care and Use of Laboratory Animals* [4.18]. Eight cyclic light horse mares (4 Quarter Horse, 4 Thoroughbred) with a median age of 8 years (range 4-16) were utilized in a crossover design during October, 2015, and July to September, 2017, in Baton Rouge, LA.

Mares were monitored by transrectal ultrasonography (SonoSite Edge, Fujifilm, Bothell, WA) throughout the study period. Mares in estrus as determined by the presence of uterine edema and with a follicle > 35 mm diameter were treated once with human chorionic gonadotropin (hCG, 2000 IU, IV, Chorulon, Merck Animal Health, Kenilworth, NJ). Mares were artificially inseminated every other day until ovulation was detected with $\geq 1 \times 10^9$ total motile spermatozoa from one stallion of known fertility. Reproductive examinations by ultrasonography were performed twice daily after insemination until detection of ovulation. After ovulation, mares were randomly assigned to one of two treatment groups: control (normal diestrus) or induced aluteal rescue (ALR). The ALR mares were treated according to a protocol previously described [4.19]. Briefly, mares were treated once daily with PGF2α (10 mg, IM, Lutalyse, dinoprost tromethamine, Zoetis, Florham Park, NJ) on Days 0 through 4 [4.19]. On Day 5 post-ovulation, mares in the ALR group were administered long acting biorelease altrenogest

(225 mg, IM; BET Pharm, Lexington, KY) [4.19]. The control group was treated on the same schedule with saline solution (2 mL. IM). Mares in both groups were evaluated for pregnancy by reproductive ultrasonography on Days 12, 13, 14, 15, 17, and 22, or until Day 14 if not pregnant, and embryonic vesicle diameter was recorded if pregnant.

Serial blood samples were collected from the time mares ovulated (Day 0) until detection of heartbeat (Days 22 or 23) if pregnant in both control and ALR groups. Plasma was harvested and stored at -20° C until assayed for progesterone. Concentrations of plasma progesterone for all cycles resulting in a pregnancy were determined by a progesterone radioimmunoassay (RIA). For the progesterone RIA, both the intraassay and interassay coefficients of variants were < 15%. A technician blinded to treatment protocols and groups performed the progesterone assays using a MP Biomedical double antibody approach as previously described [4.20].

Normality of data was confirmed and statistical significance for all data analyses was set at $P \leq 0.05$. Data for embryo vesicle diameter and concentrations of plasma progesterone were analyzed by ANOVA for repeated measures and data for pregnancy rate was analyzed by a student's t-test (Sigma Plot V14.0, Systat Software, San Jose, CA). Data are expressed as mean ± SEM.

**4.4 Results**

The overall mean progesterone concentration throughout the study duration (Day 0 to Day 23) was significantly different between groups. The ALR group remained < 1.0 ng/mL with an overall concentration of 0.86 ± 0.58 ng/mL compared to the control group overall concentration of 3.76 ± 0.54 ng/mL ($p < 0.001$). The mean daily progesterone concentration from ovulation (Day 0) to altrenogest administration on Day 5 differed

significantly in the ALR group compared to the control, 0.35 ± 0.16 ng/mL vs. 2.50 ± 0.25 ng/mL respectively ($p < 0.001$) (Fig. 4.1). The mean concentration of progesterone in the control group reached 2.43 ng/mL on Day 3 in contrast to a mean concentration of 0.69 ng/mL in the ALR group (Fig 4.1). Concentrations of plasma progesterone differed significantly between control vs. ALR group beginning on Day 4, remained so until Day 11 and was also significantly different on Days 14 and 16 ($p < 0.05$) (Fig. 4.1).

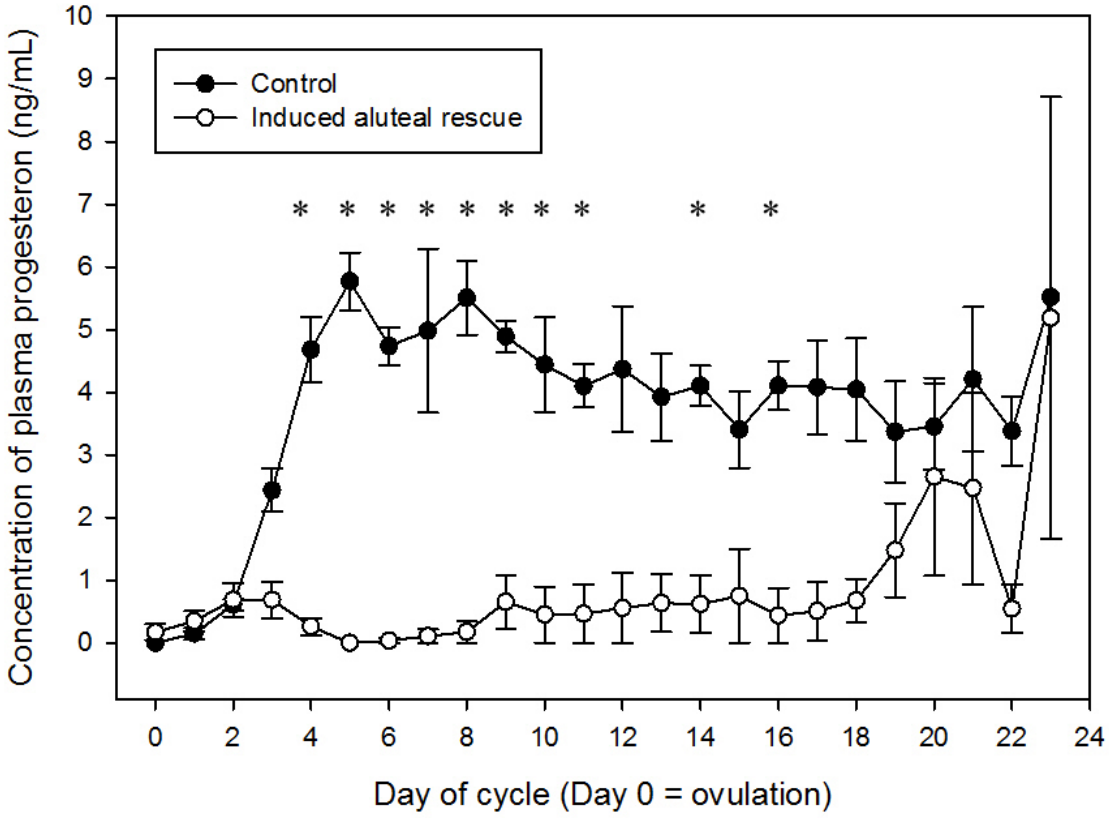


Fig. 4.1. Mean (± S.E.M.) daily concentrations of plasma progesterone in control and induced aluteal rescue treatment groups. During the induced aluteal treatment, note that serial administration of PGF2α immediately post-ovulation once daily on Days 0 through 4 caused the mean daily concentrations of plasma progesterone to remain < 1.0 ng/mL from Day 0 through Day 19. This is in contrast to the control (luteal) cycle where progesterone rose > 2.4 ng/mL on Day 3. The mean daily concentrations of plasma progesterone differed significantly in control vs. aluteal group beginning on Day 4 and remained so until Day 11 and also on Days 14 and 16 (*; $p < 0.05$)

A significant difference was observed in the pregnancy rate between the ALR group and the control group; 50% (4/8) compared to 100% (4/4) respectively ($p < 0.05$). Overall, the size of the embryonic vesicles in the AL group was smaller than the control group (Table 14., Fig. 4.2). A significant difference was observed in the size of the vesicles in the ALR group on Days 14 and 15 ($p < 0.05$) of pregnancy compared to the control group (Table 4.1, Fig. 4.2).

Table 4.1 Mean (± S.E.M.) embryonic vesicle diameter on different days post-ovulation in control and induced aluteal rescue (ALR) treatment groups. The embryonic vesicle size is smaller on all days examined in ALR vs. control, with a significant difference observed on Days 14 and 15.

| Day of cycle (Day 0 = ovulation) | ALR (mm) | Control (mm) |
|---|---|---|
| **12** | 4.55 ± 1.05 | 8.67 ± 1.33 |
| **13** | 8.03 ± 1.05 | 12.50 ± 1.04 |
| **14** | 12.01 ± 1.15[a] | 17.75 ± 1.32[b] |
| **15** | 16.10 ± 1.23[a] | 22.25 ± 0.63[b] |
| **17** | 24.50 ± 1.11 | 27.25 ± 0.48 |

a, b; $p < 0.05$

A heartbeat was detected on either Day 22 or 23 in all pregnancies (control and ALR). Two of the pregnant mares in the ALR group ovulated a secondary CL during pregnancy. One mare ovulated the secondary CL at Day 13 and one mare at Day 16.

**4.5 Discussion**

The present study evaluated the effects of progesterone-deprivation during the periovulatory period on embryo survival and early pregnancy development in the mare. Progesterone-deprived (aluteal) cycles defined by mean plasma progesterone < 1.0 ng/mL were induced in all mares subjected to serial PGF2α treatments in the ALR group from Day 0 until altrenogest administration on Day 5. Delayed embryonic development

was noted in the ALR group with overall embryonic vesicle diameter smaller than the control group on Days 12-15, and 17. The delayed vesicle size in the ALR group did catch up to the control group and heartbeats were detected on either Day 22 or 23 in both groups. This study has provided new information about progesterone deprivation during the early post-ovulatory period and its effects on early pregnancy development in the mare.

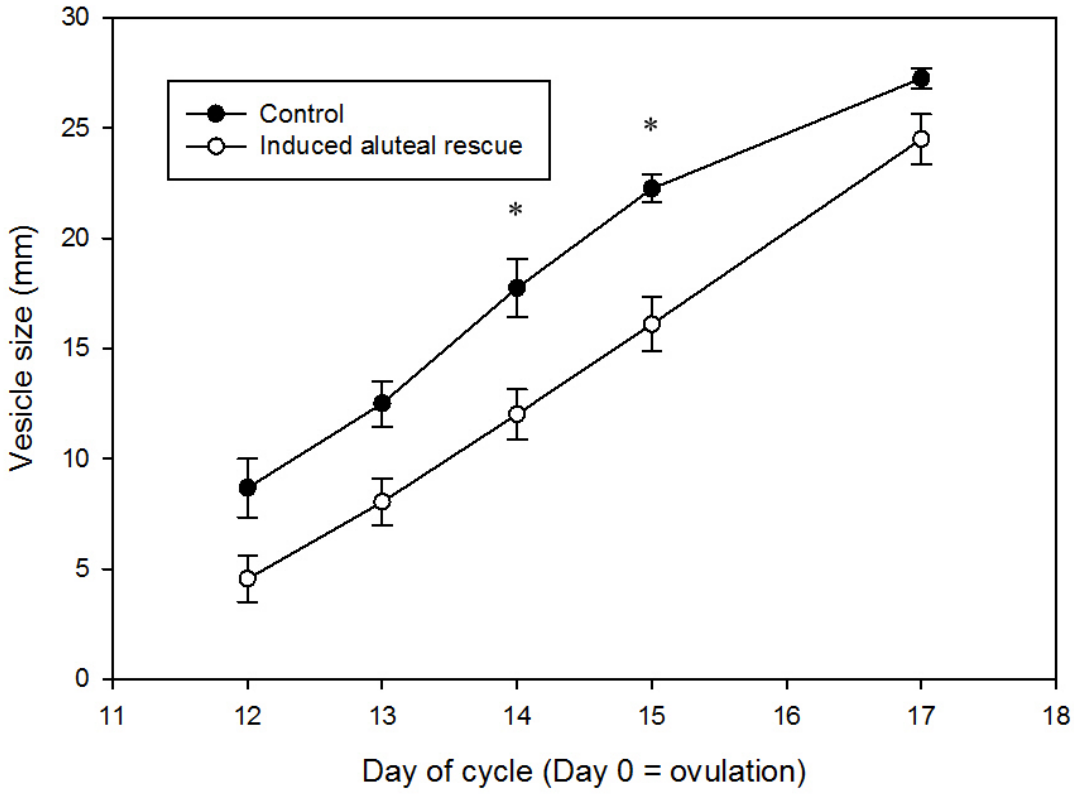


Fig. 4.2. Mean (± S.E.M.) embryonic vesicle diameter in control and induced aluteal rescue treatment groups. The diameter of the embryonic vesicle is significantly different in control vs. AL mares on Days 14 and 15. (*; $p < 0.05$)

Concentrations of plasma progesterone were significantly altered in the ALR group compared to controls. Serial administration of PGF2α once daily (antiluteogenic

treatments) altered normal CL development and function. Long acting altrenogest was administered Day 5 post-ovulation. This synthetic progestagen has a proven bioactivity for the progesterone receptor in the mare [4.21]. It has been demonstrated that progesterone is the only hormone required for the maintenance of pregnancy in a variety of ways. In one study ovariectomized mares remained pregnant after embryo transfer when supplemented only with progesterone [4.22]. Progesterone is essential for the production of uterine histotroph, the primary conceptus nutrition until placentation [4.22, 4.23]. Inadequate levels of progesterone are not compatible with pregnancy and increased embryonic loss is more likely to occur when progesterone is < 2.0 ng/mL [4.8].

A difference was observed in conceptus growth during Days 12-17 when they were deprived of progesterone during the periovulatory period. These results demonstrate that the presence of progesterone significantly affects early conceptus development in the mare. This is in agreement with studies conducted in sheep that found enhanced fetal growth when progesterone was administered during the first three days of pregnancy [4.10]. Increased embryo survival and improved development was also seen in cattle when progesterone was supplemented during the periovulatory period [4.24]. In the present study pregnancies deprived of progesterone from Day 0 to Day 5 had delayed development characterized by a smaller vesicle size than control counterparts. One study demonstrated that the expression of progesterone-mediated endometrial transcripts related to pregnancy and conceptus nutrition is affected during induced aluteal cycles [Chapter 3]. The retarded pregnancy during the present study was likely caused by the altered hormonal milieu present in the AL cycles and the subsequent effects on the uterine environment.

The equine conceptus initiates steroidogenesis as early as Day 6 with measurable amounts of progesterone, androgens, and estrogens isolated from Day 8 embryos [4.25]. At Day 12, significant quantities of estrogens are produced and this continues until Day 20 [4.25, 4.26]. These conceptus-derived estrogens act via endometrial estrogen receptors and influence uterine function in a way that remains to be elucidated [4.27]. It has been demonstrated that equine embryos produced in an *in vivo* progesterone-deprived environment are developmentally delayed and of lower quality than embryos developed during normal diestrus with elevated progesterone [4.17]. Additionally, expression of progesterone-mediated transcripts is altered when *in vivo* embryos are progesterone-deprived [Chapter 3].

The ALR cycles resulted in a significantly lower pregnancy rate compared to control cycles. Other studies in the mare have also demonstrated that pregnancy rate is affected when luteal function is disturbed during the periovulatory period [4.14-4.16]. Although this study was not able to distinguish between fertilization failure and embryo loss, a fertile stallion, young mares and a crossover design was used. Treatment mares served as controls and having achieved a 100% pregnancy rate in the control group, it is our assumption that fertilization failure is not a factor in this study. The results of the present study concur with similar studies that indicate sufficient concentrations of progesterone are essential during the periovulatory period to successfully establish pregnancy [4.14-4.16].

**4.6 Conclusions**

We have demonstrated that embryo development and pregnancy can occur in a progesterone-deprived environment. Normal embryonic growth and development were

altered, but each successful conceptus was able to reach the appropriate parameters by the time a heartbeat developed. Furthermore, pregnancy rate was significantly affected by progesterone-deprivation. Future studies to evaluate the effects of periovulatory progesterone-deprivation on the placentation and epigenetic programming of the fetus are warranted.

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# CHAPTER 5: EVALUATING THE EFFECTS OF AN INDUCED ALUTEAL CYCLE ON THE FERTILITY OF SUBSEQUENT EMBRYO COLLECTIONS

## 5.1 Summary

A 3-year-old Thoroughbred mare underwent 3 consecutive embryo collection cycles to evaluate the effects of an induced aluteal cycle on the fertility of subsequent embryo collections. In the first cycle (Cycle 1), antiluteogenic treatments were administered resulting in an aluteal cycle (daily progesterone < 1.0 ng/mL). Embryo collection was performed 8 days post-ovulation. At the time of embryo collection during Cycle 1 the mare was in estrus with 2 follicles > 35 mm and the presence of uterine edema as determined by reproductive ultrasonography. The mare was artificially inseminated the day of embryo collection and two embryos were successfully collected 8 days post-ovulation (Cycle 2). The mare was monitored for a return to estrus and artificially inseminated when a follicle ≥ 35 mm was detected. A successful embryo collection was performed 8 days post-ovulation (Cycle 3). Cycle 2 and 3 were normal (luteal) cycles demonstrated by a normal post-ovulatory rise in progesterone ranging from 8 to 30 ng/mL. The interovulatory interval between Cycle 1 and 2 was 10 days which was significantly different from the interovulatory interval between Cycles 2 and 3, which was 17 days ($p < 0.05$). This case report describes an embryo collection from a mare during an aluteal cycle followed by successful consecutive embryo collections during normal (luteal) cycles. To our knowledge, this is the first report of an embryo collection from a mare in estrus, followed by a subsequent embryo recovery after artificial insemination on the same day the previous embryo flush and recovery occurred.

## 5.2 Introduction

In the horse, embryo collections are typically performed on Days 7 or 8 after ovulation, a time when concentrations of blood progesterone are elevated [5.1, 5.2]. Blastocysts or expanded blastocysts are the developmental stages commonly identified in embryos recovered during that period [5.3]. For mares that are enrolled in programs of repeated embryo collections, a luteolytic injection of PGF2α is commonly administered shortly after embryo collection to allow a return to estrus and an opportunity for a new breeding [5.4]. The time to return to estrus and subsequent ovulation is variable because mares may be at different stages of follicle development at the time of PGF2α administration. Ovulation following PGF2α treatment may occur as early as 3 to 5 days following the luteolytic treatment or take as long as 11-13 days. This variation in ovulation is due to differences in follicle size when PGF2α is administered. On average, most mares ovulate 7 to 10 days following PGF2α treatment. Therefore, it is not unusual for several days to elapse between the day of the embryo flush and the day of the next breeding.

In the present report, we describe the successful embryo collection from a mare during an aluteal cycle followed by successful consecutive embryo collections during normal (luteal) cycles. The mare was in estrus on the day embryo collection was performed during the aluteal cycle. To our knowledge, this is the first report of an embryo collection from a mare in estrus, followed by another embryo recovery after artificial insemination on the same day the previous embryo flush and recovery occurred.

## 5.3 Materials and Methods

A 3-year-old Thoroughbred mare underwent 3 consecutive embryo collection cycles. The mare was monitored by transrectal ultrasonography (SonoSite Edge, Fujifilm, Bothell, WA). Reproductive ultrasonography was used to identify signs of estrus as follows: the presence of a follicle ≥ 35 mm diameter, marked endometrial edema and the absence of luteal tissue. All artificial inseminations were performed with ≥ 1 x $10^9$ total motile fresh spermatozoa obtained from one stallion of known fertility.

On the first estrus, the mare was artificially inseminated and treated with hCG (2000 IU, IV, Chorulon, Merck Animal Health, Kenilworth, NJ). The mare was then examined twice daily to determine the occurrence of ovulation. Once ovulation was detected (Day 0), the mare was subjected to antiluteogenic treatments (AL) as previously described [5.5, 5.6]. Briefly, PGF2α (dinoprost tromethamine, 10 mg, IM, Lutalyse, Zoetis, Florham Park, NJ) was administered twice daily on days 0, 1, and 2 and once daily on days 3 and 4. Daily blood samples were collected from breeding until the day of embryo collection on Day 8. Embryo collection was performed in an aseptic manner using Lactated Ringers Solution (LRS) with no supplementation (MWI Veterinary Supply, Boise, ID). The uterus was lavaged with 0.5 to 1 L of LRS at a time and a total of 4 L of LRS was used at each embryo collection. Once an embryo was identified it was classified according to stage of development, photographed and the diameter measured.

A morula 178 μm in diameter was collected on Day 8 post-ovulation from the first embryo collection. (Fig. 5.1). On the day the morula was collected, the mare was in estrus as determined by receptivity when teased to a stallion and reproductive ultrasonography with two preovulatory follicles measuring 41 and 35 mm in diameter,

and the presence of uterine edema 2 (scale of 0 to 4); the corpus luteum could no longer be detected. Approximately 2 hours following embryo collection, the mare was artificially inseminated and administered hCG. Two ovulations were confirmed by transrectal ultrasonography and another embryo collection was performed on Day 8 post ovulation resulting in the successful recovery of two expanded blastocysts measuring 1056 and 1031 μm (Fig. 5.1).

Immediately following this second embryo collection, the mare was treated with a single injection of PGF2α (dinoprost tromethamine,10 mg, IM; Lutalyse, Zoetis, Florham Park, NJ) and monitored for a return to estrus. Seven days after embryo collection, a 40-mm follicle was detected in the presence of uterine edema as detected by reproductive ultrasonography. On that day, the mare was artificially inseminated and administered hCG. Ovulation was detected 48 hours post-breeding and a third consecutive embryo collection was performed on Day 8, resulting in the recovery of an expanded blastocyst measuring 1510 μm (Fig 5.1).

Plasma samples were analyzed for progesterone concentrations using an automated fluorometric enzyme immunoassay analyzer (AIA 360®, Tosoh Bioscience, Inc., South San Francisco, CA), as previously described for horse plasma [5.7].

**5.4 Results**

The administration of serial PGF2α was successful in inducing an aluteal cycle as demonstrated by the daily plasma progesterone concentration during the first cycle remaining < 1.0 ng/mL during the postovulatory period (Fig. 5.2). In contrast, during the second and third cycle, the mare displayed normal luteal progesterone profiles (Fig. 5.2).

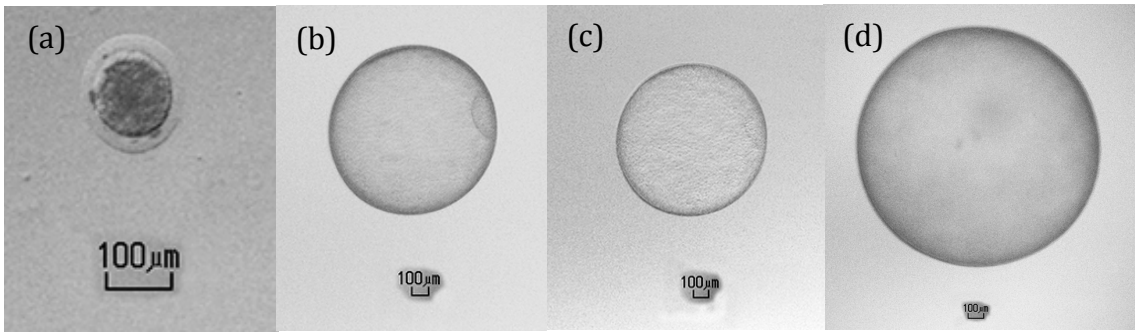


Fig. 5.1. Embryos collected 8 days post-ovulation from consecutive cycles. During Cycle 1 the embryo collected was classified as a morula and measured 178 µm in diameter (a). A synchronous double ovulation was detected during Cycle 2 and two expanded blastocysts measuring 1056 and 1031 µm in diameter were collected (b and c respectively). An expanded blastocyst 1510 µm in diameter was collected during Cycle 3, a normal (luteal) cycle (d).

In the present report, a remarkable interval of only 10 days elapsed between the first two consecutive collections of 8-day old embryos. This was significantly different than the 17 day interval between the second and third embryo collections ($p < 0.05$). This 10-day interval was possible because 1) the mare was in estrus on the day the first embryo collection was performed and she was artificially inseminated two hours after the successful embryo collection, and 2) she ovulated within 48 hours of hCG administration.

**5.5 Discussion**

As previously reported, serial administration of PGF2α beginning within 12 hours of ovulation prevented normal corpus luteum function as confirmed by reproductive ultrasonography and daily concentrations of plasma progesterone remaining < 1 ng/mL [5.5, 5.6]. In agreement with our first report describing that early embryonic development can take place in an aluteal environment, a Day 8 embryo was recovered during an aluteal

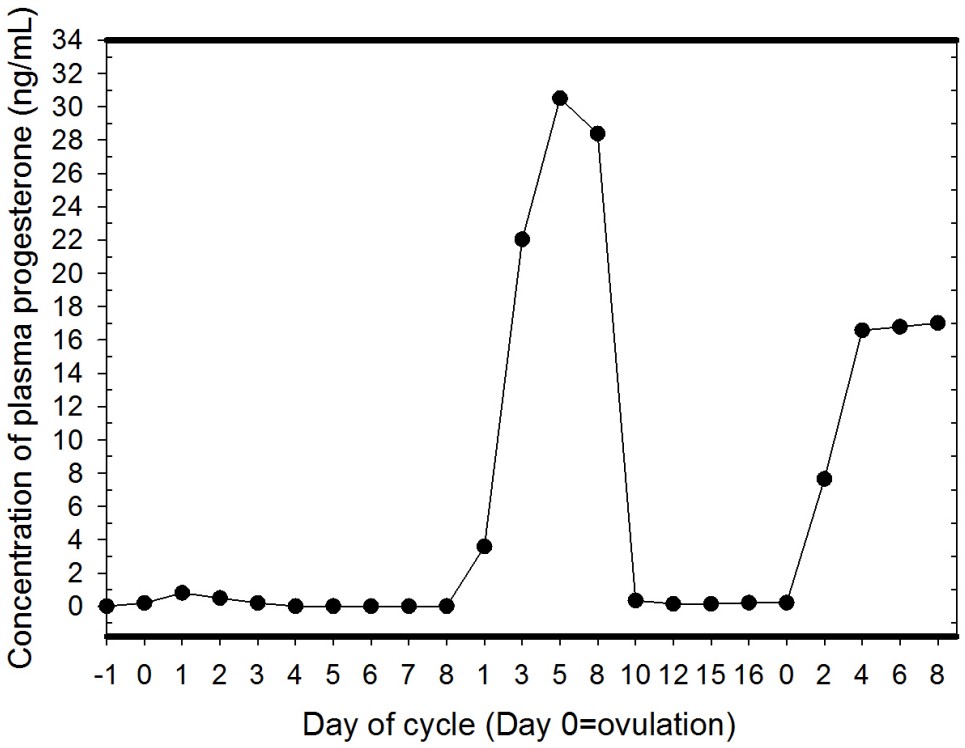


Fig. 5.2. Mean (± S.E.M.) daily concentrations of plasma progesterone (ng/mL). During cycle 1, the induced aluteal cycle, there was no rise in plasma progesterone with daily concentrations remaining < 1.0 ng/mL. This is in contrast to cycles 2 and 3, the normal (luteal) cycles, where a post-ovulatory rise in progesterone was observed. The elevated plasma progesterone concentration was sustained during the diestrus period until PGF2α was administered on the day of embryo collection (Day 8).

cycle in the present report [5.5]. The embryo was classified as a morula, denoting marked delayed development. Its viability could not be ascertained as the aluteal embryo was processed and frozen for future analysis. In a previous study, 4 mares that were artificially inseminated and underwent serial PGF2α treatments following ovulation were treated with injectable altrenogest on Day 6 after ovulation in an attempt to rescue a potential developing pregnancy [5.8]. Two mares became pregnant and heartbeats were detected at Day 22 in both pregnancies [5.8]. In the present report, the embryo collected during the aluteal cycle was developed in a progesterone-deprived environment and the

mare was already in estrus on the day of embryo collection. It is plausible to speculate that the viability of that embryo was significantly compromised. Therefore, the viability of aluteal embryos on Day 8 after ovulation remains to be determined by embryo collection and transfer to synchronized luteal recipient mares or following rescue by administration of exogenous progestagens as described in our previous report [5.8].

Equine practitioners have reported the effect of repeated breeding and embryo flushes on the fertility of embryo donor mares. In one report, 27 embryos were recovered from 70 embryo flushes, yielding an embryo recovery rate of 38.6% [5.1]. In the subsequent year, 15 embryos were produced in 21 embryo flushes, resulting in 71% embryo recovery rate [5.1]. The authors concluded that repeated breeding and embryo collection attempts did not affect the fertility of embryo donor mares [5.1]. In another study, 21 mares were subjected to repeat artificial inseminations and embryo flushes (average of 7 per mare) [5.9]. In this study, the breeding and embryo transfer procedures were associated with increased chronic inflammation but the future fertility of those mares was not investigated [5.9]. In the present report, four embryos were recovered during three consecutive embryo flushes. Three morphologically normal, grade 1 expanded blastocysts were recovered in the two luteal cycles following the first aluteal cycle. These results are in agreement with our previous reports that showed the potential for pregnancy or embryo production remains the same for mares ovulating and subjected to antiluteogenic treatments and for mares artificially inseminated in the post-aluteal cycle [5.5, 5.6]. Interestingly, as previously reported, the collection of an endometrial biopsy during estrus does not affect the likelihood of pregnancy [5.10]. In the present report, two endometrial biopsies were taken immediately after embryo collection and

approximately 2 hours prior to artificial insemination. Embryos were collected in the subsequent cycle, illustrating how the collection of an endometrial biopsy during estrus does not seem to affect the chance for pregnancy in mares artificially inseminated on that cycle [5.10].

**5.6 Conclusions**

To our knowledge, this is the first report of an embryo collection from a mare in estrus, followed by artificial insemination the same day and subsequent embryo collection occurring 10 days after the previous embryo collection. Further investigation is warranted to utilize serial treatments of PGF2α applied at different times during the periovulatory period to shorten the interovulatory interval and embryo collection efficiency in the mare. Additionally, investigation of the viability of embryos produced in an aluteal environment can be evaluated utilizing embryo transfer.

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# CHAPTER 6: SUMMARIZING DISCUSSION AND CONCLUSIONS

## 6.1 Discussion

A novel in vivo model was utilized to evaluate the effects of progesterone deprivation on the Day 8 embryo and endometrium. Additionally, embryos developed in a progesterone-deprived environment were subsequently rescued by administration of altrenogest to evaluate the effects of early progesterone deprivation on normal pregnancy establishment. The administration of serial doses of PGF2α resulted in mean plasma progesterone < 1.0 ng/mL in all AL groups (Chapters 2-5). This contrasted greatly with control groups, which displayed a typical diestrus rise up to 10 ng/mL and created a clearly altered hormonal milieu in control vs. AL groups. The morphological and molecular characterization of Day 8 embryos in AL vs. control cycles demonstrated that lack of progesterone adversely affects embryo quality and alters the expression of certain genes in both the embryos and endometrium (Chapter 2 and 3). The AL cycles subsequently rescued by altrenogest administration significantly affected pregnancy rate and normal pregnancy development (Chapter 4). The administration of serial doses of PGF2α significantly decreased the interovulatory interval (IOI) and had no effect on future fertility with the successful collection of three embryos from two subsequent normal diestrus cycles after an AL cycle (Chapter 5).

The model utilized in the present studies is an established model that administers serial doses of PGF2α within 12 hours of ovulation to prevent normal luteal function (antiluteogenesis) in the mare [6.1-6.6]. This model reliably results in mean plasma progesterone < 1.0 ng/mL throughout the duration of the typical diestrus period in the mare. When the established model administering 8 serial doses of PGF2α was utilized in

this work, aluteal cycles, as defined by mean plasma progesterone < 1.0 ng/mL, were induced and maintained in all treated cycles throughout the study period (Day 0 to Day 8) (Chapters 2-4). When the protocol was modified to administer PGF2α once daily for 4 days beginning within 12 hours of ovulation, aluteal cycles were induced in all treated mares from Day 0 to the time of altrenogest administration on Day 5 (Chapter 5).

Embryos produced during AL cycles were affected compared to control embryos produced during normal diestrus (high progesterone) cycles (Chapter 2). It has been established that the equine embryo expresses non-genomic progesterone receptors as early as Day 7 [6.7]. The effects of the altered hormonal milieu were detected in the morphology of the Day 8 AL embryos as well as in the gene expression. The majority of the Day 8 AL embryos collected were significantly smaller in diameter, earlier in development, and lower in quality grade. Typically, the stage of embryo recovered at a Day 8 embryo collection would be an expanded blastocyst visible to the naked eye measuring between 500-1000 μm [6.8]. The AL embryos were classified as morula and early blastocysts with diameters between 151-195 μm compared to the expanded blastocysts between 600-1122 μm in diameter in the control group.

The transcript expression of AL embryos and endometrium was affected (Chapter 3). The AL embryos had a significantly increased expression of four genes *ESR1, P19, APOB* and *PGR*. It has been described that steroid hormones mediate the expression of *ESR1, PGR* and *P19* [6.7, 6.9, 6.10]. The upregulation of these transcripts is likely due to the lack of progesterone during AL cycles which mimics estrus vs. diestrus. The upregulation of *APOB*, a transcript related to nutrient transport and embryo development, complements the results of the developmentally delayed and lower quality AL embryos

[6.11]. The upregulation of this transcript may be an attempt to increase nutrient uptake and compensate for nutrient deficiencies experienced during progesterone-deprivation. In the endometrium four transcripts were upregulated, *FGF9, sPLA2, PGR, ESR1,* and four transcripts were downregulated, *HK2, P19, IFNE, CTGF*. Steroid hormones drive the expression of *sPLA2, PGR, ESR1, HK2, P19, FGF9* and *IFNE* [6.9, 6.10, 6.12-6.25]. The altered expression of these transcripts is likely due to the absence of progesterone during induced aluteal cycles. The remaining transcript, *CTGF,* is a growth factor implicated in the maintenance of early pregnancy in the mare [6.26]. It is likely that the endometrial development in a progesterone-deprived environment during AL cycles negatively affected the signaling events required for the appropriate expression of *CTGF*.

It was demonstrated that embryos deprived of progesterone during the early preimplantation period (Day 0 to Day 4) and rescued by the administration of altrenogest displayed delayed embryonic vesicle growth overall compared to controls as characterized by a smaller diameter on Days 12-15 and 17 (Chapter 4). However, the rescued AL pregnancies were able to establish normal pregnancy parameters as determined by the detection of a heartbeat at Days 22 or 23. This study is in agreement with other studies that sufficient concentrations of progesterone are essential during early the early postovulatory period to establish pregnancy [6.27, 6.28]. The AL cycles also resulted in a significantly lower pregnancy rate compared to control cycles. Other studies in the mare have also demonstrated that pregnancy rate is affected when luteal function is disturbed during the periovulatory period [6.27, 6.28].

It has been demonstrated that the application of antiluteogenic treatments results in a shortened IOI with no effect on the fertility of subsequent cycles [6.1, 6.6].

Remarkably, a 10-day interval was recorded between two consecutive embryo collections after an induced aluteal cycle (Chapter 5). Additionally, the two successful embryo collections occurred during the cycles immediately following an induced aluteal cycle. Conflicting reports exist regarding repeated embryo collections in the mare. Some practitioners report an increase in chronic inflammation and others see no difference on the fertility of donor mares [6.29, 6.30]. In the present study four embryos were collected from three consecutive cycles indicating there was no effect on fertility when antiluteogenic treatments and repeated embryo collections were employed in a young, fertile mare.

**6.2 Conclusions**

The present studies characterize the importance of adequate progesterone levels beginning immediately post-ovulation and continuing throughout the early preimplantation period and early pregnancy in the mare. The results of this work demonstrate that progesterone is a key mediator in early embryonic growth and development. Progesterone plays an important role in the preparation of the endometrium so pregnancy can be established and maintained. The effects of progesterone on the preimplantation embryo may be both direct via non-genomic steroid receptors and indirect via the improperly primed endometrium and inadequate uterine histotroph. Furthermore, although early pregnancies were affected by progesterone-deprivation, the administration of an exogenous progestagen rescued the pregnancy and the developing conceptus was able to reach normal pregnancy parameters up to Day 23. The lasting effects of progesterone deprivation on the embryo and pregnancy viability have yet to be determined. Future studies transferring progesterone-deprived embryos to synchronized

luteal recipients may offer additional information. Additionally, modifications of the protocol used to induce the aluteal cycles may be applied to shorten the interovulatory interval and increase reproductive efficiency of embryo donor mares.

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## APPENDIX: LETTER OF PERMISSION FROM ELSEVIER FOR CHAPTER 2

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# VITA

Chelsey Leisinger, a native of Cedar Falls, Iowa, received her B.S. in Animal Science from Iowa State University in 2007. Upon graduation she worked as a bovine embryo transfer technician in Waverly, Iowa before moving to Columbus, Ohio in 2009. During her time in Ohio she worked at The Ohio State University College of Veterinary Medicine as the Theriogenology Research Assistant and Clinical Coordinator from 2009 to 2013. While working she attained her M.S. degree in Veterinary and Comparative Medicine from The Ohio State University College of Veterinary Medicine in 2013. After receiving her M.S. degree, Chelsey left Ohio to begin working as an embryologist at a human fertility clinic in Overland Park, KS. She remained there until 2014 when she moved to Baton Rouge, Louisiana to begin her doctorate program in Biological and Veterinary Medical Sciences at Louisiana State University School of Veterinary Medicine. After receiving her Ph.D. and completing her High-complexity Clinical Laboratory board examination with the American Association of Bioanalysts, Chelsey will be the Laboratory Director at Fertility Answers of Baton Rouge and Lafayette in Louisiana.